Universidad Autónoma de Madrid
Departamento de Bioquímica

Estudio de la regulación epigenética y rutas de señalización celular implicadas en respuesta a quimioterapia con cisplatino en cáncer no microcítico de pulmón

Tesis doctoral

María Cortés Sempere
Madrid, 2012

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Directoras de la tesis
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Por la presente, **Rosario Perona Abellón,** Doctora en Farmacia y Profesor de Investigación del CSIC en el Instituto de Investigaciones Biomédicas Madrid, y **Mª Inmaculada Ibáñez de Cáceres,** Doctora en Biología e Investigador "Miguel Servet" contratado por la Fundación para la Investigación Biomédica del Hospital de la Paz, INGEMM-HULP y del Instituto de Investigación Sanitaria IdiPAZ, certifican que:

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"Estudio de la regulación epigenética y rutas de señalización celular implicadas en respuesta a quimioterapia con cisplatino en cáncer no microcítico de pulmón" en el Instituto de Investigaciones Biomédicas "Alberto Sols".

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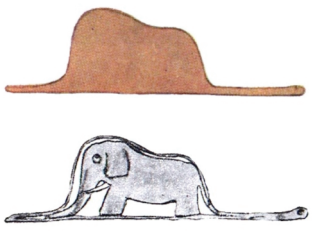
Director de la Tesis
Profesor de Investigación
CSIC

Dra. Mª Inmaculada Ibáñez de Cáceres
Director de la Tesis
Investigador Contratado
INGEMM-IdiPAZ-HULP

A mis padres

A mi hermana



Antoine de Saint-Exupéry, **El principito.**

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RESUMEN

El cáncer no microcítico de pulmón (CNMP) comprende más de un 80% de los casos de cáncer de pulmón, con 1,2 millones de casos nuevos en el mundo cada año. El cisplatino (CDDP), se utiliza como tratamiento estándar en este tipo de cáncer en estadios avanzados en combinación con otros agentes quimioterápicos, sin embargo esta terapia dista de dar los resultados esperados debido al frecuente desarrollo de resistencia. Los mecanismos moleculares por los cuales se desarrolla la resistencia a CDDP no están claros, pero se creen multifactoriales, entre ellos se encuentran numerosos eventos moleculares y cambios genéticos y epigenéticos. Una mayor comprensión de este proceso de resistencia podría ayudar al desarrollo de biomarcadores que predigan resistencia a CDDP en CNMP, con lo que se podría conseguir la identificación de tratamientos personalizados para los pacientes llevando a una reducción tanto de la mortalidad como de efectos secundarios innecesarios. Con el objetivo de profundizar en el conocimiento de los mecanismos de regulación de la expresión génica y vías de señalización implicadas en el proceso de resistencia a CDDP el trabajo se dividió en dos bloques en los que se estudió, por una parte los cambios epigenéticos mediados por la exposición a CDDP y por otra, las vías de señalización en las que están involucradas las proteínas MKP1, PI3K/AKT y NFκB, también relacionadas con la resistencia a dicho agente quimioterápico. Así en primer lugar observamos que la metilación del promotor de *IGFBP-3* induce resistencia a CDDP, y que este proceso ocurre a través de la activación de la vía de supervivencia de IGF-IR/PI3K/AKT en células tumorales. Además existe una fuerte correlación entre el estado de metilación del promotor de *IGFBP-3* y la respuesta a CDDP en muestras de pacientes de CNMP. También se observó que los pacientes en estadio I que no presentan el promotor de *IGFBP-3* metilado muestran mayor supervivencia libre de enfermedad que aquellos con el promotor metilado y por último, que la conjugación de los datos sobre el estado de metilación del gen *IGFBP-3* junto con el estado de activación de los receptores IGF-IR y EGFR así como de la proteína AKT en muestras de pacientes, puede permitirnos predecir con una alta especificidad y exactitud si éstas responderán o no a CDDP. En segundo lugar, hemos observado que además de la vía de señalización de PI3K/AKT, las vías de NFκB y la función de la fosfatasa MKP1 también están implicadas en la respuesta a CDDP en CNMP. Además se ha observado en muestras de pacientes de CNMP que un 20% de éstas presentan la expresión de p65/RelA y RelB en el núcleo, y que las líneas tumorales que tenían mayor activación de la vía de NFκB son las más sensibles al fármaco bortezomib. Extrapolando estos datos a la clínica nos indicarían que tal vez aquel porcentaje de pacientes de CNMP que presentasen NFκB activo en sus biopsias podrían beneficiarse del tratamiento con bortezomib, y que probablemente no respondan o respondan pobremente a la quimioterapia con CDDP. Unificando los resultados globales de esta tesis, observamos que la activación de las rutas estudiadas (PI3K/AKT y NFκB), los bajos niveles de expresión de *IGFBP-3* y los altos niveles de la proteína MKP1 corresponden con una alta IC_{50} en cinco líneas tumorales de CNMP analizadas. Finalmente, en este trabajo hemos descrito diferentes biomarcadores de predicción de resistencia a CDDP, que podrían ser utilizados para un tratamiento personalizado en pacientes con CNMP, resultando en un mayor beneficio clínico.

ABSTRACT

Non-small-cell lung cancer (NSCLC) comprises more than 80% of lung cancers, with 1.2 million new cases worldwide each year. Cisplatin (CDDP)-based chemotherapy remains the standard treatment in combination with other chemotherapeutic agents; however, NSCLC patients frequently exhibit chemotherapy resistance. The molecular mechanisms of drug resistance remain unclear and are believed to be multifactorial, involving host factors, numerous molecular events and genetic and epigenetic changes. A better understanding of the resistance process could be useful for the development of biomarkers to predict CDDP resistance in NSCLC patients and for the implementation of personalised treatments, thus decreasing mortality and reducing unnecessary side effects. In order to gain insight into the gene expression changes and the signalling pathways involved in the resistance process, this study has been divided into two blocks: the first comprises the study of the epigenetic changes mediated by CDDP exposition and the second is focused on the signalling pathways involving the MKP1, PI3K/AKT and NFκB proteins, which are related to CDDP resistance. We first observed that *IGFBP-3* promoter methylation induces CDDP resistance, and this process occurs through IGF-IR/PI3K/AKT pathway activation in tumour cells. In addition, there is a strong correlation between *IGFBP-3* promoter methylation status and the CDDP response in NSCLC patient samples. In addition, it was observed that those patients diagnosed with pathological stage I, whose tumours harbour an unmethylated promoter, had a clearly increased disease-free survival compared with methylated stage I patients. Finally, the integration of the *IGFBP-3* methylation data together with the activation status of IGF-IR and EGFR and the protein AKT in NSCLC samples, allows us to predict if these patients would or would not respond to CDDP with high accuracy and precision. Secondly, we observed that in addition to the PI3K/AKT signalling pathway, the NFκB pathway and MKP1 protein functions are involved in the CDDP response in NSCLC. In addition, 20% of the studied NSCLC samples expressed nuclear p65/RelA and RelB. On the other hand, it was observed that cells with high basal NFκB activity were more sensitive to bortezomib. In clinical practice, these results may indicate that NSCLC patients with an active NFκB in their biopsies could benefit from treatment with bortezomib, and that they would likely not respond or would respond poorly to CDDP treatment. Overall, we observed that activation of the signalling pathways PI3K/AKT and NFκB, *IGFBP-3* low expression levels and high levels of MKP1 protein correlated with a high IC_{50} in five NSCLC cell lines analysed. Finally, in this study we have described various predictive biomarkers of CDDP response that could be used to personalise treatment for NSCLC patients.

ÍNDICE

ABREVIATURAS

5-AZAdC: 5-aza-2 deoxicitidina
ADNc: ADN codificante
AKT: Homólogo al oncogén viral de timoma murino v-akt
ALK: Quinasa del linfoma anaplásico
AP1: La proteína activadora 1
APC: Adenoatous Polyposis Coli
ASK: Quinasa reguladora de la señal de apoptosis
ATF2: El factor de transcripción activador 2
BAX: Proteína X asociada a Bcl2
BCL2: Leucemia linfocítica crónica/ linfoma de células B 2
BRAF: Homólogo B1 del oncogen viral del sarcoma murino v-raf
BRCA1: Cáncer de mama tipo 1
BRC: Proteína de la región de fractura
CDH13: Cadherina 13
CDK: Quinasas dependientes de ciclina
CDKN2A: Inhibidor 2 a de la quinasa dependiente de ciclina
DAPK: Proteína quinasa asociada a muerte 1
DLK: Proteína homóloga a delta (Drosophila)
DUSP: Proteínas fosfatasas duales y específicas
EML4: Proteína 4 similar a la proteína asociada a microtúbulos de equinodermos
ERCC: Subunidad de la reparación por escisión de nucleótidos
FDA: Administración de medicamentos y alimentos de Estados Unidos
FAS: Superfamília del receptor del factor de necrosis tumoral
HER2: Receptor del factor de crecimiento epidérmico en humanos 2
hMLH1: Proteína de reparación del ADN de la vía de reparación de bases mal apareadas
HSF1: Factor de transcripción del choque térmico 1
IGFBP: Proteínas de unión a IGF
IKK: Quinasa del inhibidor de NFκB
KRAS: Oncogén homólogo al virus del sarcoma de rata Kirsten
MET: Receptor del factor de crecimiento de hepatocitos
MGMT: O-6-metilguanina-DNA metiltransferasa
MLK: Quinasas de linaje mixto
MLL: Leucemia de linaje mixto o mieloide/linfoide
MRP: Proteína asociada a la resistencia a múltiples drogas
MYC: Oncogén homológo a mielocitomatosis (aviar)
NEMO: Modulador esencial de NFκB
NFATc1: Componente citosólico del complejo de transcripción del factor nuclear de células T activadas
NRAS: Oncogén homólogo al virus del sarcoma de rata de neuroblastoma
PTEN: Homólogo de fosfatasa y tensina
RAS: Oncogén homólogo al virus del sarcoma de rata
RASF1: Miembro de la familia con un dominio de asociación a Ras 1
RB: Retinoblastoma
RISC: Complejo de inhibición de la expresión inducido por ARN

STK11: Serina treonina quinasa 11
TAK: Quinasa activada por el factor de crecimiento tumoral ß
TAO: Proteína quinasa del aminoácido 201
TGM2: Transglutaminasa 2
TIMP3: Inhibidor de la metalopeptidasa 3
TSA: Tricostatina
XIAP: Inhibidor de la apoptosis ligado a X

INTRODUCCIÓN

1. EL CÁNCER

El cáncer es un conjunto de enfermedades en las cuales se produce un crecimiento celular anormal e incontrolado. La tumorogénesis es la consecuencia de un proceso secuencial que incluye alteraciones genéticas y epigenéticas (activación de oncogenes y silenciamiento de genes supresores de tumores) que llevan a una modificación progresiva de las células normales en células transformadas. Este proceso es análogo a la evolución Darwiniana, en el cual estas alteraciones confieren a la célula ciertas ventajas en cuanto a la supervivencia y capacidad de crecimiento. Se han descrito hasta más de 100 tipos de cáncer, que poseen cambios genéticos y epigenéticos muy diferentes unos de otros, pero que presentan alteraciones esenciales en 8 mecanismos que regulan la fisiología celular para poder dar lugar a un tumor maligno, éstas son:

- Autosuficiencia en señales de crecimiento
- Insensibilidad a la señales inhibitorias de crecimiento
- Evasión de la apoptosis
- Potencial replicativo ilimitado
- Angiogénesis
- Invasión de tejidos y metástasis
- Reprogramación del metabolismo energético
- Evasión de la destrucción por el sistema inmune

Estas dos últimas alteraciones celulares son facilitadas por dos características derivadas de la neoplasia como son la inflamación, la inestabilidad del genoma y diversas mutaciones (Hanahan and Weinberg, 2011).

El cáncer es la principal causa de muerte a escala mundial. Se le atribuyen 7,6 millones de defunciones (aproximadamente el 13% del total) ocurridas en todo el mundo en el año 2008. Los tipos de cáncer que más muertes causan cada año son los de pulmón, estómago, hígado, colon y mama. Se prevé que las muertes por cáncer sigan aumentando en todo el mundo y alcancen la cifra de 13,1 millones en 2030 (OMS2008).

1.1 EL CÁNCER DE PULMÓN

El cáncer de pulmón fue el tipo de cáncer más diagnosticado y la principal causa de muerte a nivel mundial en hombres en el año 2008. Para las mujeres fue el cuarto tipo de cáncer más diagnosticado y la segunda causa de muerte (OMS2008). En España, en el año 2005, fallecieron por cáncer de pulmón 19115 personas, de las cuales 16645 eran varones frente a 2470 mujeres (Escuin, 2009). Las diferencias en la incidencia del cáncer de pulmón entre sexos o entre los diferentes países parecen estar ligadas al hábito tabáquico. El consumo de tabaco es el factor de riesgo más importante, y es la causa del 22% de las muertes mundiales por cáncer en general, y del 71% de las muertes mundiales por cáncer de pulmón (OMS2008).

1.1.1 Clasificación del cáncer de pulmón y su tratamiento

Desde el punto de vista histológico el cáncer de pulmón puede dividirse en dos tipos: el cáncer microcítico de pulmón (CMP) y el cáncer no microcítico de pulmón (CNMP), este último representa un 80-85% de los cánceres de pulmón. Dentro del cáncer de pulmón no microcítico, se pueden distinguir otros tres subtipos dependiendo del tipo celular al que se diferencien histológicamente, y son:

1. **Carcinoma epidermoide o de células escamosas:** Compuesto por células epiteliales con diferenciación escamosa e identificable por la producción de islas de queratina.
2. **Adenocarcinoma:** Conformado por células con diferenciación glandular o con producción de mucina similares a las células calciformes que integran la mucosa de cualquier epitelio.
3. **Carcinoma de células grandes:** Células grandes, anaplásicas y poligonales, con núcleos vesiculares. No se puede clasificar ni como epidermoide ni como adenocarcinoma.

Casi la totalidad (99%) de los cánceres de pulmón son carcinomas. De éstos, en España, el carcinoma epidermoide o de células escamosas es el más frecuente, seguido del adenocarcinoma. Sin embargo, aunque con diferencias temporales según las zonas geográficas, hay una tendencia universal al aumento de la proporción de adenocarcinomas y a la disminución de epidermoides, tendencia que ya se registró a partir de los años setenta en EE.UU., donde los primeros son hoy, con mucho, la variedad más común. En general, esta estirpe es la que tiene un vínculo menos estrecho con el tabaquismo; así la proporción de adenocarcinomas entre no fumadores varía en distintos países entre el 40 y el 76% (Escuin, 2009). En España, los datos del IARC (Agencia Internacional de Investigación del Cáncer) 2007 Volumen IX, ponen de manifiesto la siguiente distribución de los diferentes subtipos histológicos del CNMP: carcinoma de células escamosas 41,4%, adenocarcinoma 22,1% y carcinoma de células grandes 16,3%.

El tratamiento del CNMP depende de la clasificación que se le da al tumor del paciente. Para clasificar dichos tumores se utiliza el sistema TNM, en el cual T define la extensión del tumor primario, N a la afectación de ganglios linfáticos (tamaño y número) y M a la metástasis a otros órganos (Rami-Porta et al., 2009). Utilizando este sistema los tumores se clasifican en: Estadio 0, se define como carcinoma in situ, se encuentran células cancerosas en el revestimiento de las vías respiratorias. Estadio I, el tumor se encuentra únicamente en el pulmón, está rodeado por tejido normal y no existe afectación ganglionar ni a distancia, dependiendo del tamaño del tumor se diferencia entre IA (2-3cm) y IB (≤ 5cm). Estadio II, se subdivide en IIA, el tumor es ligeramente más grande (5-7cm) y no se ha diseminado a ganglios linfáticos cercanos o es un tumor más pequeño (2-5cm) que se ha diseminado a los ganglios linfáticos cercanos, y el subtipo IIB, el tumor mide de 5-7cm y se ha diseminado a los ganglios linfáticos o es un tumor más grande que puede o no haber invadido estructuras cercanas del pulmón, pero que no se ha diseminado a los ganglios linfáticos. Es importante diferenciar entre el subtipo IIA, potencialmente operable, del estadio IIB sin posibilidades reales de cirugía, pues la aproximación terapéutica es totalmente distinta. Estadio III, el tumor mide más de 7cm o se ha extendido a la pared torácica o al diafragma cerca del pulmón; o el cáncer se ha diseminado a los ganglios linfáticos mediastínicos, subcarinales o más allá de estos. Por último el Estadio IV, en el que el tumor ha metastatizado en otros órganos.

Dependiendo de esta clasificación, el tratamiento varía desde la cirugía a combinaciones de cirugía más quimioterapia; quimioterapia más cirugía o quimioterapia/radioterapia o quimioterapia más radioterapia (Tabla1). Actualmente, la quimioterapia en CNMP se basa en la combinación de cisplatino o carboplatino y otros fármacos cómo vinorrelbina, gemcitabina, paclitaxel, docetaxel, etopósido, e irinotecán. Aunque también se han incorporado tratamientos dirigidos contra dianas específicas, como son el receptor de EGF, la quinasa ALK y agentes que inhiben el proceso angiogénico, como los inhibidores de VEGF-A.

ESTADIO	T	N	M	TRATAMIENTO
0	Tis	N0	M0	· Cirugía
IA	T1a-T1b	N0	M0	· Cirugía + Quimioterapia (ensayos clínicos)
IB	T2a	N0	M0	
IIA	T1a—2a T2b	N1 N0	M0	· Quimioterapia + Cirugía ó Cirugía + Quimioterapia · Cirugía+radioterapia (ensayo clínico)
IIB	T2b T3	N1 N0	M0	
IIIA	T3 T1a-3 T4	N1 N2 N0-1	M0	· Quimioterapia + Cirugía ó Cirugía + Quimioterapia · Cirugía + Quimioterapia+radioterapia · Cirugía+radioterapia
IIIB	T4 Cualquier T	N2 N3	M0	· Quimioterapia+radioterapia · Quimioterapia + Cirugía · Radioterapia (paliativo) · Nuevas combinaciones (ensayos clínicos)
IV	Cualquier T	Cualquier N	M1a-M1b	· Quimioterapia combinada · Quimioterapia combinada+terapia dirigida (Ac monoclonales) · Terapia dirigida inhibidores tirosina quinasa · Radioterapia (paliativo) · Nuevas terapias (ensayos clínicos)

Tabla 1. Clasificación y tratamiento del CNMP

El cisplatino (CDDP) es un agente que reacciona con el ADN produciendo uniones covalentes intra e intercatenarias entre guaninas adyacentes (Eastman, 1985). Estas lesiones activan varias rutas de transducción de señales, entre ellas aquellas relacionadas con reconocimiento de daño y reparación del ADN, así como parada del ciclo celular y apoptosis (Siddik, 2003). Como muchos agentes quimioterápicos, el CDDP induce la activación de las quinasas del extremo N-terminal de c-Jun (JNK) y p38, y del factor de transcripción nuclear κB (NFκB) (Sanchez-Perez et al., 2000, Sanchez-Perez et al., 1998). La activación de JNK por CDDP tiene lugar a través de la ruta MEKK1/SEK1, y está directamente relacionada con la muerte celular (Sanchez-Perez and Perona, 1999).

1.1.2. Resistencia a quimioterápicos

La resistencia a la qiumioterapia es uno de los problemas principales que presenta el tratamiento del cáncer de pulmón. La resistencia puede ser intrínseca (propia del tumor) o adquirida (desarrollada a lo largo del tratamiento con el quimioterápico). La causa más común de la adquisición de resistencia a quimioterápicos en general es la expresión de uno o más transportadores que detectan y expulsan las drogas anticancerígenas de las células. Otros mecanismos de resistencia, incluyen la evasión de la apoptosis inducida por la quimioterapia y la inducción de mecanismos de detoxificación (Gottesman, 2002).

En el caso del CDDP, existen varios factores implicados en la resistencia del tumor al mismo, estos son:

- **Unión insuficiente del CDDP al ADN.** En muchos tumores con resistencia adquirida a CDDP se observa una acumulación de platino reducida en comparación con las células parentales (Kelland et al., 1992). El CDDP es una molécula muy polar que entra en las células relativamente despacio en comparación con otras moléculas utilizadas para el tratamiento del cáncer. La entrada de CDDP en la célula está influenciada por las concentraciones de los iones de sodio y potasio; por el pH; por la presencia de agentes reductores y por la acción de transportadores y canales, que se unen al mecanismo de difusión pasiva (Gately and Howell, 1993). Entre los transportadores de membrana implicados en el transporte de cobre, el transportador de cobre 1, CTR1, se ha visto involucrado en el mecanismo de entrada del CDDP. Otro transportador importante en resistencia a CDDP son los transportadores dependientes de ATP implicados en el mecanismo de resistencia a múltiples drogas (MDR). Tanto MDR1, MRP1, MRP3 y MRP5 como las ATPasas ATP7A y ATP7B, se han visto relacionadas con la expulsión de CDDP de la célula. El CDDP, también puede conjugarse con glutatión, proceso catalizado por las enzimas glutatión-S-transferasas (GSTs), para luego ser más fácilmente exportado por las bombas transportadoras conjugadas con glutatión-S dependientes de ATP (MRP1 y MRP2). Por último las metalotioneinas, cuya expresión aumenta en resistencia a CDDP están implicadas en la unión a metales pesados y su detoxificación (Kelland, 2007).

- **La resistencia posterior a la unión al ADN.** Una vez que el CDDP se ha unido al ADN, la célula puede sobrevivir por activación de los mecanismos de reparación del ADN o por tolerancia al daño genético. La principal vía de reparación del ADN en resistencia a CDDP es la de reparación de escisión de nucleótidos (NER). Tanto en diferentes líneas celulares como en muestras de pacientes se ha descrito una relación entre el aumento en los niveles de expresión de la endonucleasa ERCC1 y resistencia a CDDP. También es importante la ruta de reparación de bases mal apareadas (MMR), la pérdida de la actividad de esta vía resulta en una inhibición de la apoptosis y por tanto en resistencia a CDDP (Kelland, 2007).

- **Alteración de rutas implicadas en supervivencia y apoptosis.** A parte de los mecanismos descritos anteriormente, en respuesta al CDDP se activan diferentes rutas involucradas en apoptosis y supervivencia celular. La alteración de estas rutas por mutaciones en proteínas que forman parte de las mismas pueden dar lugar al desarrollo de resistencia a CDDP. En los últimos años también se ha observado una relación entre el tratamiento con CDDP y la metilación del ADN. El CDDP puede inducir metilación *de novo* en el ADN provocando la represión de la expresión de determinados genes implicados en la respuesta al fármaco (Koul et al., 2004, Chang et al., 2010). Estas alteraciones se describirán más detenidamente en otro apartado de esta memoria.

2. REGULACIÓN EPIGENÉTICA EN CANCER

La epigenética es la ciencia que estudia los cambios heredables en la función génica que se producen sin un cambio en la secuencia del ADN. Los procesos epigenéticos tienen un papel fundamental en fenómenos fisiológicos como son la embriogénesis, la impronta y la inactivación del cromosoma X, pero también en el desarrollo de enfermedades, entre ellas el cáncer (Robertson, 2005). Los cambios epigenéticos se pueden producir por diferentes mecanismos, como son la metilación del ADN, la modificación post-traduccional de histonas y los micro ARNs.

2.1. MODIFICACIÓN DE HISTONAS

El ADN se dispone alrededor de las proteínas histonas para formar los nucleosomas. Las histonas H2A, H2B, H3 y H4 se agrupan en dos dímeros de H2A-H2B y un tetrámero de H3-H4 para formar un nucleosoma. Las modificaciones de histonas consisten en reacciones covalentes que afectan a sus regiones amino-terminal. Estas modificaciones post-traduccionales incluyen la acetilación, la metilación, la fosforilación, la ubicuitinación, la sumoilación, y la ADP-ribosilación y pueden tener efectos directos sobre diferentes procesos nucleares, incluyendo la transcripción génica, la reparación y replicación del ADN y la organización de cromosomas (Kouzarides, 2007). Estas modificaciones ocurren en aminoácidos específicos, los más estudiados son la metilación y la acetilación de lisinas en las histonas H3 y H4. Las enzimas que catalizan estas reacciones son las histona acetiltransferasas (HAT), las desacetilasas de histonas (HDAC), las histonas metiltransferasas (HMT) y las histonadesmetilasas (HDMT). Éstas pueden funcionar como activadores o represores transcripcionales dependiendo del residuo sobre el que actúen. Generalmente la acetilación de histonas está asociada con la activación transcripcional, en cambio la metilación de histonas depende del tipo de aminoácido que se metile y de su posición (Taby and Issa, 2010).

En cáncer, la modificación más común de histonas es la reducción en la acetilación de la lisina 16 de la histona H4 (Fraga et al., 2005). La pérdida de acetilación es mediada por las HDACs y éstas se encuentran sobreexpresadas o mutadas en diferentes tipos tumorales (Ropero et al., 2006, Zhu et al., 2004). Además en algunos tipos de cáncer (colon, útero, pulmón y leucemia) se han observado translocaciones que dan lugar a la formación de proteínas aberrantes, mutaciones o deleciones de HAT y de genes relacionados con HAT, contribuyendo a una acelitación aberrante de histonas (Portela and Esteller, 2010).

2.2. MICRO ARNs

Los micro ARNs (miARNs) son ARNs no codificantes de 20 a 22 nucleótidos, que por complementariedad con la región 3’ UTR de los ARNs mensajeros llevan a su degradación o a una represión de la traducción y por lo tanto a la inhibición de la expresión génica (Bartel, 2009). El ADN que codifica estos miARNs se encuentra normalmente localizado en la regiónes intrónicas o intergénicas (Rodriguez et al., 2004), aunque también puede derivar de secuencias genómicas repetitivas (Smalheiser and Torvik, 2005). Los miARNs son sintetizados por la enzima ARN polimerasa II como pri-miARNs, que posteriormente son procesados a pre-miARNs en el

núcleo. Los pre-miARNS son exportados al citoplasma donde se modifican para dar un ARN maduro de doble cadena. Finalmente una de las cadenas es seleccionada como miARN maduro y la otra es degradada. El miARN maduro se incorpora entonces al complejo de silenciamiento inducido por ARN (RISC) para así seleccionar y unirse al ARN mensajero diana (Garzon et al.). Un sólo miARN pueden tener como diana cientos de ARN mensajeros que pueden estar implicados en numerosos procesos biológicos incluidos diferenciación, ciclo celular, y apoptosis (Iorio and Croce, 2009). La primera evidencia de la correlación entre cáncer y miRNAs apareció en el año 2002 cuando se observó que el miR-15a y el miR16-1 estaban codificados por una región frecuentemente delecionada del cromosoma 13, en leucemia linfocítica crónica de células B (Cimmino et al., 2005). Actualmente se conocen miARNs que se expresan de forma aberrante o que están mutados en una gran variedad de tumores, lo cual sugiere su implicación en iniciación y progresión del cáncer.

2.3 METILACIÓN DEL ADN

La metilación del ADN en mamíferos consiste en la adición de un grupo metilo a citosinas situadas adyacentes a guaninas, los llamados dinucleótidos CpG. Esta reacción es catalizada por las ADN metiltransferasas (DNMTs). La DNMT1 es considerada la responsable del mantenimiento de esta modificación epigenética, mientras que las DNMT3A y DNMT3B son metiltransferasas *de novo* y pueden completar el proceso de metilación y corregir errores (Jones and Liang, 2009). En los últimos años se ha descrito un nuevo proceso para la desmetilación del ADN. Éste lo llevan a cabo las proteínas TET (ten eleven translocation protein) que catalizan la conversión de la 5-metilcitosina (5mC) a 5-hidroximetilcitosina (5hmC), este nuevo nucleótido parece ser un intermediario en la desmetilación del ADN. A partir de este se puede producir una desmetilación pasiva (a través de la replicación) o una desmetilación activa a través de la glicosilación del nucleótido y otras enzimas, o a través de las proteínas TET; con la transformación de la 5hmC a otros intermediarios (5-carboximetilcitosina y 5-formilmetilcitosina)(Williams et al., 2011).

En el genoma existen pequeñas regiones de 0,5 a 4Kb de longitud conocidas como “islas CpG”, enriquecidas en este dinucleótido y que representan un 1% del total del genoma. Muchas de estas islas se encuentran en las regiones promotoras de casi la mitad de los genes del genoma de mamíferos y generalmente se encuentran no metiladas en células adultas. La metilación del ADN reprime la transcripción, directamente, impidiendo la unión de factores de transcripción específicos, e indirectamente, por reclutamiento de proteínas de unión a los dinucleótidos metilados CpG (MBDs) que regulan la estructura de la cromatina reprimiendo la transcripción. La presencia de anomalías en la metilación del ADN dan lugar al desarrollo de diferentes enfermedades en humanos, entre ellas el cáncer (Jaenisch and Bird, 2003, Jones and Baylin, 2002).

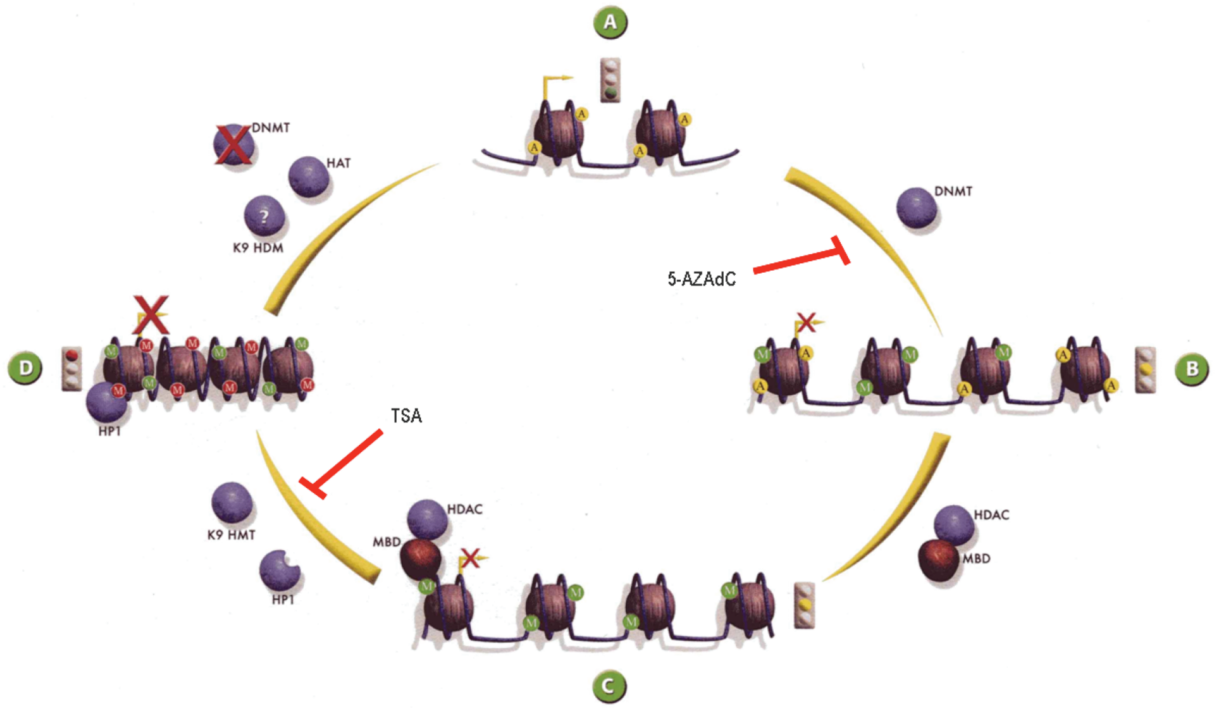

Figura 1. Esquema de silenciamiento de la expresión génica a través de mecanismos epigenéticos. A) Ejemplo de cromatina transcripcionalmente activa en la que las histonas se encuentran acetiladas y las islas CpG del promotor no metiladas. B) La metilación de determinadas citosinas de la isla CpG a través de la acción de las DNMTs impide parcialmente la unión de factores de transcripción, reprimiendo la expresión del gen. Este mecanismo puede ser revertido artificialmente por el compuesto 5-AZAdC, que actúa incorporándose en cada nueva molécula de ADN sintetizada, durante el proceso de replicación, donde forma enlaces covalentes con los sitios activos de la metiltransferasa, inhibiendo su actividad enzimática, lo que resulta en una desmetilación generalizda del ADN. C) La metilación del ADN provoca la unión de proteínas que tienen dominios de unión al ADN metilado (MBDs), que a su vez pueden llevar asociadas HDACs que retiran los grupos acetilos de las histonas, dando lugar a que la cromatina tome una conformación cerrada. Este proceso puede ser revertido por el compuesto TSA, un agente inhibidor de las desacetilasas de histonas capaz de revertir la estructura cerrada de la cromatina, mediante la acumulación de histonas acetiladas, lo que restablecería la expresión del gen. D) Se produce la metilación de las histonas en residuos específicos que dan lugar a un total silenciamiento de la cromatina. Finalmente se puede producir una vuelta a la activación de la transcripción mediante la acción de las HDMTs, de las HATs y de las TET.

2.3.1. Metilación y cáncer

La primera vez que se demostró una relación entre la metilación del ADN y el cáncer fue en 1983, cuando se observó que las células tumorales tenían un grado de metilación menor que las células parentales (Feinberg and Vogelstein, 1983). Este fenómeno está relacionado con una pérdida de metilación en las regiones repetitivas del genoma provocando inestabilidad cromosómica en estas células tumorales. Sin embargo el principal evento que esta involucrado en el origen de un gran número de cánceres es la hipermetilación de islas CpG en los promotores de genes supresores de tumores (TSGs) (Herman and Baylin, 2003). Estos genes pueden mediar diferentes funciones celulares implicadas en el desarrollo del cáncer, como son la regulación del ciclo celular, la reparación del ADN, la interacción célula-célula, la apoptosis y la angiogénesis (Esteller, 2008).

Hoy en día, no hay duda de que alteraciones en la metilación del ADN tienen un papel muy importante en la tumorogénesis. Una metilación aberrante de las islas CpGs de genes específicos, es un mecanismo epigenético tan común como las mutaciones puntuales o pérdidas de heterozigosicidad (LOH) que causan el silenciamiento de los TSGs en cánceres humanos (Ibanez de Caceres and Cairns, 2007).

En el cáncer de pulmón están afectados por la hipermetilación los promotores de determinados genes que se saben relacionados con cáncer, ya que numerosos estudios ilustran las diferencias entre los niveles de metilación en tumores y en tejido normal adyacente y la asociación con datos clínicos. Entre los diferentes genes alterados por la metilación en cáncer de pulmón encontramos, *RASSF1, CDKN2A, CDH13, APC, MGMT, DAPK, IGFBP-3 y TIMP3* (Brock et al., 2008).

En los últimos años ha surgido la evidencia de la relación entre cambios epigenéticos y el desarrollo de resistencia a quimioterápicos, tanto *in vitro* como *in vivo.* La primera alteración epigenética que se asoció con la sensibilidad a una droga quimioterápica fue la metilación del gen *MGMT* y la sensibilidad a agentes alquilantes. A partir de este estudio se han encontrado otras correlaciones entre genes metilados y resistencia/sensibilidad a quimioterápicos, por ejemplo un 15% del cáncer colorrectal muestra inestabilidad de microsatélites debido a la metilación del promotor del gen *hMLH1,* que codifica una enzima de reparación. Clínicamente, los tumores de cáncer colorrectal con metilación en dicho gen son menos agresivos pero no responden a 5-fluoracilo. Por otro lado la inactivación epigenética del gen *FANCF,* que codifica una proteína implicada en la reparación del ADN, está asociada a la sensibilidad a agentes que dañan el ADN como el CDDP y la mitomicina C. Además la restauración de la expresión de *FANCF* en líneas celulares de cáncer de ovario mediante el uso del agente desmetilante del ADN 5-AZAdC, induce resistencia a CDDP. Por otra parte, la metilación del promotor del gen *CHFR,* que codifica la proteína con actividad ligasa E3, se asocia con sensibilidad a inhibidores de la polimerización de microtúbulos en cáncer gástrico, y la del gen *CDK10,* que da lugar a la quinasa dependiente de ciclina 10, con sensibilidad a tamoxifeno en cáncer de mama (Toyota et al., 2009).

3. VÍAS DE SEÑALIZACIÓN IMPLICADAS EN CÁNCER DE PULMÓN Y EN RESISTENCIA A FÁRMACOS

Se han encontrado un gran número de rutas de señalización alteradas en el proceso de tumorogénesis en cáncer de pulmón. El 98% de las líneas de cáncer de pulmón tienen alteraciones en uno de estos genes: *TP53* (79%), *CDKN2A* (59%), *RB1* (35%), *STK11* (27%), *MYC* (20%), *KRAS* (17%), *PTEN* (11%), *PIK3CA* (8%), *EGFR* (7%), *NRAS* (6%), *MET* (5%), *BRAF* (2%), *HER2* (2%), *AKT* (0%), y *EML4-ALK* (0%). En CNMP encontramos sobre todo mutaciones en los genes *TP53, KRAS, EGFR, HER2, c-MET, LKB1, PIK3CA, BRAF* y *EML4-ALK,* asociadas también a resistencia a quimioterapia (Reungwetwattana et al., 2012).

3.1. Receptores tirosina quinasa

Los receptores con actividad tirosina quinasa pertenecen a la familia de proteínas tirosina quinasa, y su estructura cuenta con un dominio catalítico intracelular tirosina quinasa, un dominio transmembrana y un dominio extracelular de unión a ligandos. Muchos de estos receptores se activan tras la unión de ligandos específicos, como factores de crecimiento y citoquinas. La unión del ligando permite la dimerización y subsiguiente activación del dominio catalítico citoplasmático por la trans-fosforilación de tirosinas. Esta fosforilación aumenta su actividad tirosina quinasa creando nuevos sitios de unión para proteínas diferentes, que finalmente inducen la fosforilación

de proteínas citoplasmáticas, traduciendo por tanto la señal extracelular a través de la membrana plasmática (Hunter, 2009). Se han identificado mas de 58 receptores tirosina quinasa, entre ellos, el receptor de insulina (IR), el receptor tirosina quinasa parecido a FMS (FLT3), el receptor cKit, el receptor del factor de crecimiento epidérmico (EGFR), el receptor del factor de crecimiento derivado de plaquetas (PDGFR), el receptor del factor de crecimiento de fibroblastos (FGFR), el receptor del factor de crecimiento vascular-endotelial (VEGFR), el receptor del factor estimulante de colonias 1 (CSF1R) y el receptor del factor de crecimiento parecido a insulina (IGF-IR). La actividad de estos receptores es esencial para el mantenimiento de la homeostasis celular y la modulación de la expresión génica en varias rutas de señalización intercelulares e intracelulares. Las vías de señalización más relevantes, activadas por los receptores tirosina quinasa incluyen las vías de Ras/ERK/MAPK, PI3K/AKT y JAK/STAT. Debido a la importancia de las rutas activadas por los receptores tirosina quinasa, la sobreexpresión o mutaciones de tipo activadoras de éstos son frecuentes en cáncer. Por ello se han desarrollado una serie de fármacos que impiden la señalización de estos receptores, como son los inhibidores de la actividad tirosina quinasa (TKI) y los anticuerpos monoclonales humanizados. Los TKI son inhibidores de bajo peso molecular que bloquean la unión de ATP a los dominios tirosina quinasa de los receptores impidiendo por tanto su fosforilación (Krause and Van Etten, 2005). Algunos de estos TKI han sido aprobados para el tratamiento de determinados cánceres, como por ejemplo gefitinib (Iressa™) y erlotinib (Tarceva™), inhibidores de EGFR aprobados para el tratamiento de CNMP. Los anticuerpos monoclonales utilizados en clínica para inhibir la actividad de receptores tirosina quinasa pueden reconocer tanto el receptor como el ligando impidiendo la unión de ambos y así la activación del receptor. Por ejemplo, en CNMP, se utiliza en clínica en combinación con quimioterapia el cetuximab (Erbitux™) (Krause and Van Etten, 2005).

3.1.1. EGFR

EGFR (también conocido como ErbB-1) pertenece a la familia de receptores humanos de crecimiento epidérmico. A su dominio extracelular se unen diferentes ligandos como el factor de crecimiento epidérmico (EGF) o el factor de crecimiento transformante beta (TGFβ), provocando su dimerización y por tanto su activación, que a su vez media la activación de vías de señalización implicadas en proliferación celular, migración, adhesión, inducción de angiogénesis e inhibición de la apoptosis (Steins et al., 2010). Además EGFR se encuentra sobreexpresado en diferentes tipos de cánceres humanos incluyendo pulmón, cabeza y cuello, colon, páncreas, mama, ovario, vejiga y riñón y gliomas (Normanno et al., 2006). Más del 60% de los pacientes con CNMP muestran sobreexpresión de EGFR. Además de la sobreexpresión de este receptor se han encontrado diferentes mutaciones activadoras del dominio quinasa del receptor en CNMP. Las mutaciones más frecuentes consisten en pequeñas deleciones en el exón 19 y una mutación puntual en el exón 21 (L858R) las cuales suman el 90% de mutaciones en el dominio quinasa de EGFR. Otras mutaciones en el exon 18 y 20 se encuentran en el restante 10% de casos. Los pacientes con deleciones en el el exon 19 son los que mejor responden a inhibidores de EGFR. En contraste a estas mutaciones se encontró una mutación secundaria de un cambio de base que induce un cambio de treonina a metionina (T790M) en el exón 20 que confiere resistencia a los inhibidores de EGFR (Zhang et al., 2010).

3.1.2. IGF-IR

IGF-IR funciona como un heterotetrámero formado por dos subunidades α extracelulares donde se une el ligando IGF-I y dos subunidades β que contienen los dominios transmembrana y tirosina quinasa. Una vez el ligando se une al receptor se produce la activación de los dominios quinasa, lo que provoca el reclutamiento y subsecuente fosforilación de diferentes proteínas, como la proteína que contiene el dominio 2 de homología a Src (Shc) o las proteínas sustrato del receptor de insulina 1-4 (IRS 1-4), que a su vez inducen la propagación de la señal a través de las vías PI3K/AKT y MAPK implicadas en la proliferación celular y la inhibición de la apoptosis. IGF-IR tiene una gran homología con el receptor de insulina (IR), e incluso puede formar receptores híbridos con las cadenas α y β de IR-A e IR-B (Hartog et al., 2007). IGF-IR une tanto IGF-I como IGF-II con gran afinidad. Un aumento en la intensidad de señalización de IGF-IR se asocia a un mayor riesgo y agresividad en cáncer. Se ha observado un aumento en la expresión de IGF-IR en algunos cánceres humanos como en el de mama, el colorrectal, el de próstata y en CNMP asociándose en varios estudios al estadio del tumor, a una menor supervivencia o a un aumento en la sensibilidad a quimioterapia cuando IGF-IR es inhibido. Existen diferentes ensayos clínicos con inhibidores tirosina quinasa específicos de IGF-IR y con anticuerpos monoclonales; sin embargo, el principal problema de estos ensayos, es la gran homología entre el receptor de insulina e IGF-IR, lo que puede provocar problemas de hiperglicemia, por ello se sigue investigando en la obtención de moléculas más específicas para su uso terapéutico (Camidge et al., 2009). Además de los efectos mitogénicos y antiapoptóticos de la vía de señalización de IGF-IR, ésta también se ha visto implicada en resistencia a quimioterápicos (Guix et al., 2008).

3.2. El eje IGF/IGFBP-3

El eje de señalización del factor de crecimiento parecido a la insulina (IGF) tiene un papel muy importante en crecimiento y desarrollo en humanos. Además, también está implicado en otros procesos fisiológicos y patológicos como en mitogénesis, angiogénesis, transformación, diferenciación, homeostasis tisular, antiapoptosis y motilidad celular (LeRoith and Roberts, 2003). IGF-I e IGF-II son dos ligandos que pueden unirse a los receptores IGF-IR, IGF-IIR e IR. En humanos, se ha demostrado a partir de estudios epidemiológicos una asociación entre niveles circulantes altos de IGF-I y un aumento en el riesgo de sufrir algunos tipos de cáncer, como por ejemplo, cáncer de vejiga, colorrectal, ovario, mama, pulmón y próstata. IGFBP-3 pertenece a una familia de proteínas de unión a IGF compuesta por 6 miembros llamados IGFBPs. Un 99% del IGF circulante se encuentra unido a estas IGFBPs, del cual una gran mayoría lo constituye un complejo de 150 KDa entre IGFBP-3 y la subunidad ácido lábil (ALS). Este complejo prolonga la vida media de IGF-I de 10 minutos a 15 horas. Las IGFBPs impiden la unión de IGF a IGF-IR, ya que IGF tiene mayor afinidad por IGFBP que por su receptor (Grimberg, 2003). Por esta razón las proteínas IGFBPs son de gran importancia en el control del ciclo celular, transformación y supervivencia, ya que impiden la activación de la vía IGF/IGF-IR/PI3K/AKT, que está muy implicada en estos procesos (Heidegger et al., 2011).

Los niveles de la proteína IGFBP-3 pueden estar regulados en cáncer por cambios en su expresión o por su degradación por proteasas. En cáncer de próstata, el antígeno específico

de próstata (PSA), que se utiliza como marcador tumoral, es capaz de degradar la proteína IGFBP-3. También se han observado niveles altos de proteólisis de IGFBP-3 en plasma de mujeres en estadios avanzados de cáncer de mama (Grimberg, 2003). Además, *IGFBP-3,* ha sido referido por diferentes autores como un gen supresor de tumores, bajo regulación epigenética en diferentes tipos de tumores como en pulmón, ovario, hígado y riñón (Ibanez de Caceres et al., 2006, Hanafusa et al., 2002, Fraga et al., 2004).

En CNMP se ha observado una relación entre niveles bajos de expresión de *IGFBP-3,* provocados por la hipermetilación del promotor, y una pobre prognosis en pacientes en estadio I (Chang et al., 2002a, Chang et al., 2002b).

3.3 La vía de PI3K/AKT

La vía de fosfoinosítido-3 quinasa (PI3K)/AKT está implicada en transformación, proliferación, supervivencia y metástasis en muchos tipos de cáncer, incluyendo el CNMP (Vivanco and Sawyers, 2002). Las proteínas PI3K de clase I son unos complejos heterodiméricos, cada uno de ellos compuesto por una subunidad catalítica y una subunidad reguladora (Wymann and Pirola, 1998). PI3K fosforila a la molécula fosfatidil inositol 4 fosfato (PIP) y 4,5 difosfato (PIP2) para convertirla en fosfatidil inositol 3,4,5 trifosfato (PIP3), éste recluta a la proteína AKT/proteína quinasa B(PKB) a la membrana citoplasmática. Este mecanismo está regulado por una proteína supresora de tumores, la fosfatasa PTEN, que invierte la fosforilación realizada por PI3K, finalizando la señal (Vivanco and Sawyers, 2002, Li et al., 1997). La activación de AKT induce la fosforilación de ciertas proteínas que median la supervivencia celular. Por ejemplo, la fosforilación tanto de IκB como del dominio de transactivación de p65 por AKT lleva a la activación de NFκB (Ghobrial et al., 2005), que promueve supervivencia por su actividad antiapoptótica. También fosforila a Bad y a la caspasa 9 (dos proteínas proapoptóticas) inactivándolas, bloqueando así la señalización de apoptosis.

La alteración de esta vía de señalización suele ser debida a mutaciones activadoras en el gen *PIK3CA.* El análisis de estas mutaciones revelan un aumento de la señal de PI3K, estimulando la señalización a través de AKT, y promoviendo así un crecimiento independiente de factores de crecimiento e incrementando la invasión celular y metástasis (Hafsi et al., 2012). No son muy frecuentes las mutaciones en el gen de *PI3KCA* en cáncer de pulmón (de un 1 a un 4% en CNMP), sin embargo, la amplificación de este gen se ha observado en un 33% de carcinomas de células escamosas y en un 5,9% de adenocarcinomas (Solomon and Pearson, 2009). Se han descrito mutaciones en el gen supresor tumoral *PTEN* en un gran numero de cánceres humanos, en cambio en CNMP tanto las mutaciones como las deleciones de *PTEN* no son muy frecuentes (5%) (Forgacs et al., 1998). Sin embargo, si lo es encontrar niveles bajos de dicha proteína o la ausencia de la misma, que podrían ser explicados por la hipermetilación del promotor, lo cual ocurre de un 25 a un 40% de los casos (Soria et al., 2002).

La frecuencia de mutaciones en *AKT* en CNMP es de un 1% (Hafsi et al., 2012) sin embargo la fosforilación de AKT se encuentra entre un 50 y un 70% de los casos de CNMP, indicando que la activación de esta ruta es un evento frecuente en esta enfermedad. Esta activación de AKT

suele deberse tanto a cambios genéticos de componentes que se encuentran por encima en la ruta de PI3K/AKT como por mutaciones en *EGFR* o por el aumento del número de copias, por mutaciones en *KRAS,* mutación o amplificación de *PIK3CA* o por pérdida de *PTEN* (Solomon and Pearson, 2009).

3.4. LA VÍA DE LAS MAPK

Las proteínas quinasas activadas por mitógenos (MAPK) median la señalización asociada a un gran número de actividades celulares, entre ellas, la proliferación, la diferenciación, la supervivencia, la apoptosis y la transformación. En mamíferos la familia de las MAPK consta de las quinasas reguladas por señales extracelulares ERK1/2, ERK3/4, ERK5, ERK7/8, y de las proteínas quinasas reguladas por estrés, p38 (isoformas α,β,γ y δ) y JNK 1/2/3 (quinasas del extremo amino terminal de cjun). Las MAPK se activan por fosforilación de los residuos serina y treonina que se encuentran en una secuencia conservada T-X-Y en el dominio de activación de la quinasa. Las vías de señalización de las proteínas MAPK están compuestas por un módulo de tres quinasas que se activan por fosforilación en cascada, teniendo como componentes cada módulo de señalización: una MAPK quinasa quinasa (MAP3K), una MAPK quinasa (MAP2K), y una MAPK. Anormalidades en la señalización de las MAPK juegan un papel crítico en el desarrollo y progresión del cáncer (Dhillon et al., 2007).

3.4.2. La vía de señalización de ERK

La vía clásica de ERK ha sido asociada a la habilidad de las células cancerígenas para crecer de forma independiente a las señales normales de proliferación y se encuentra alterada en un 30% de cánceres humanos. Se han encontrado anormalidades oncogénicas en los componentes que se encuentran por encima de la MAPK ERK en su vía de señalización, tales como la sobreexpresión o la aparición de mutaciones activadoras en los receptores tirosina quinasa, las mutaciones activadoras en Ras GTPasa y mutaciones en la MAP3K BRAF (Keyse, 2008).

3.4.3. MAPK activadas por estrés

Las células cancerígenas están expuestas a condiciones de estrés, incluyendo hipoxia, separación del sustrato, inflamación y estrés metabólico asociado a la desregulación de la producción de energía. Además se suma a esto el estrés genotóxico y farmacológico durante la quimioterapia o radioterapia. Normalmente sus efectos son anti-proliferativos y pro-apoptóticos, pero depende del contexto celular (Dhillon et al., 2007).

- **La vía de señalización de JNK**

  La vía de JNK es activada predominantemente por citoquinas, radiación UV, por falta de factores de crecimiento, por agentes que dañan el ADN, ciertos receptores acoplados a proteínas G y suero. JNK puede ser fosforilada y activada por las quinasas MEK4 y MEK7, que a su vez pueden ser activadas por diferentes MAP3K como MEKK1-4, MLL2 y 3, YTpl-2, DLK, TAO1 y 2, TAK1 y ASK1 y 2. Una vez JNK es fosforilada viaja al núcleo donde puede fosforilar

diferentes factores de transcripción, como a su principal sustrato c-jun, o a otros como ATF-2, NFATc1, HSF-1 y STAT-3. Además también puede fosforilar a p53 aumentando su actividad transcripcional y estabilizándola (Dhillon et al., 2007). A la quinasa JNK se le han atribuido papeles contrapuestos, ya que está implicada tanto en la inducción de apoptosis como en el aumento de la supervivencia celular y proliferación, y esto se debe a que JNK puede activar a un gran número de sustratos dependiendo de estímulos específicos, del tipo celular y de aspectos temporales (Bode and Dong, 2007). Por ejemplo, una activación sostenida de JNK está asociada a la activación de la apoptosis, como ocurre por ejemplo en células tratadas con CDDP (Sanchez-Perez et al., 1998).

- **La vía de P38**

  En mamíferos, p38 es activada por estrés ambiental y por citoquinas inflamatorias. P38 puede ser fosforilada y activada por MEK3 y MEK6, las cuales son activadas por diferentes MAP3K dependiendo del estímulo, como TAK1, ASK1/2, DLK, MEKK4, TAO1/2/3 y MLK2/3. Una vez activada también viaja al núcleo donde fosforila a sus diversos sustratos. P38 tiene un papel muy importante en cáncer ya que puede funcionar como un gen supresor tumoral. Además esta quinasa está relacionada con la inducción de apoptosis y ha sido descrita como un proteína reguladora negativa del ciclo celular, ya que está implicada en la activación de p53 y en la apoptosis mediada por p53 (Dhillon et al., 2007). Muchos agentes quimioterápicos requieren la actividad de p38 para inducir apoptosis, como por ejemplo el CDDP (Hernandez Losa et al., 2003).

3.4.4. MKP1

MKP1 pertenece a la familia de las MKPs, fosfatasas duales y específicas que actúan como reguladores negativos de la actividad de las MAPK. En mamíferos podemos encontrar 10 MKPs que pueden subdividirse en tres grupos dependiendo de su especificidad por el sustrato y de su localización celular. MKP1 es el miembro de la familia de las MKPs mejor estudiado, su localización celular es nuclear y tiene mayor afinidad por p38 y JNK que por ERK. El gen que codifica para MKP1 *(DUSP-1)* es un gen inmediato temprano regulado transcripcionalmente por estímulos mitogénicos, inflamatorios, y por daño en el ADN (Lau and Nathans, 1985, Charles et al., 1992). En varios tipos de tumores, como el CNMP, carcinoma de ovario y cáncer de próstata se han encontrado niveles elevados de MKP1 (Srikanth et al., 1999, Denkert et al., 2002, Wang et al., 2003). Nuestro grupo ha demostrado que MKP1 tiene un papel importante en el crecimiento tumoral y en la respuesta al tratamiento con CDDP en células de CNMP (Chattopadhyay et al., 2006). Se establecieron líneas estables de CNMP que expresaban un ARN interferente para MKP1, y se observó que estas células eran más sensibles a CDDP y que su crecimiento era más lento que el de las células parentales cuando eran inyectadas en ratones desnudos. Además, los compuestos químicos que inhibían la actividad de MKP1, inducían sensibilidad a CDDP. Estas propiedades eran específicas de MKP1 ya que la inhibición o sobreexpresión de MKP2, una fosfatasa con alta homología a MKP1, no alteraba el crecimiento del tumor ni la respuesta a CDDP en dichas células (Chattopadhyay et al., 2006).

Estos resultados sugieren que MKP1 es una diana terapéutica importante, ya que su inhibición o inactivación disminuye el crecimiento tumoral y sensibiliza a las células cancerígenas al tratamiento con CDDP. Por ello un mayor entendimiento de la función de MKP1 podría ayudar a mejorar la respuesta celular a los agentes quimioterápicos.

3.5. NFκB

La familia del factor de transcripción NFκB está compuesta por una serie de proteínas (p105, p100, p50, p52, c-Rel, RelB, RelA/p65) que forman heterodímeros y actúan como factores de transcripción reguladores de las respuestas inmune e inflamatoria (Collart et al., 1990, Libermann and Baltimore, 1990, Zhang et al., 1990). Además la activación de esta ruta de señalización protege a la célula de la apoptosis inducida por agentes quimioterápicos y por el tratamiento con citoquinas (Baldwin, 2001). En condiciones basales, los miembros de la familia NFκB forman heterodímeros en el citoplasma donde se encuentran asociados a una familia de moléculas inhibitorias conocidas como IκBs (Finco and Baldwin, 1995). Existen dos rutas de activación para NFκB, una es la vía canónica o clásica (Bonizzi and Karin, 2004), en ella el mecanismo de activación de NFκB implica la fosforilación de las IκBs mediante un complejo macromolecular, formado por IκB quinasas (IKKs, IKKα e IKKβ) y una subunidad reguladora (IKKγ o NEMO). Las IκBs, mayoritariamente IκBα, son fosforilados en dos residuos serina importantes para su actividad (Ser32 y Ser36), provocando la ubiquitinación de IκB y su degradación por el proteasoma 26S (Brown et al., 1995, Traenckner et al., 1995, DiDonato et al., 1996). Esto permite que los dímeros de proteínas NFκB puedan translocarse al núcleo y allí estimular la expresión de sus genes diana. En la vía no canónica, el complejo p100-RelB es activado por un homodímero de IKKα, que fosforila la región c-terminal de p100, provocando la ubiquitinación y degradación de dicha región, generándose un nuevo dímero, p52-RelB, que podrá translocarse al núcleo para llevar a cabo su función como factor de transcripción (Dejardin et al., 2002). Además existen diferentes estímulos que pueden activar la transcripción mediada por NFκB a través de mecanismos independientes de la translocación nuclear, como aquel que implica la fosforilación del dominio de transactivación de la subunidad p65 (Schmitz et al., 1995).

NFκB tiene un papel muy importante en el control del crecimiento celular, diferenciación y apoptosis (Kucharczak et al., 2003). La activación de MEKK1 por CDDP aumenta por un lado la muerte celular por inducción de la expresión del ligando Fas a través de AP1 y de manera paralela activa también NFκB, activación modulada por c-Jun, el principal sustrato de JNK. En ausencia de expresión de c-Jun, las células son más resistentes a la acción del CDDP, relacionándose con un incremento en la transcripción dependiente de MEKK1-NFκB (Sanchez-Perez et al., 2002).

Estos datos sugieren que los agentes quimioterápicos como el CDDP activan las rutas proapoptóticas a través de la transcripción del ligando Fas dependiente de c-Jun, y al mismo tiempo regulan de forma negativa las rutas de supervivencia, como a XIAP-1, cuya transcripción depende de NFκB. En diversos tipos de cáncer (incluyendo cáncer de mama, colon, próstata, y linfomas) NFκB es activo de forma constitutiva y está localizado en el núcleo (Nakanishi and Toi, 2005). Por ello han surgido terapias antitumorales que tienen como diana el bloqueo de la

actividad de NFκB para así inhibir el crecimiento del tumor e inducir la sensibilización de las células tumorales a la quimioterapia (Nakanishi and Toi, 2005).

El bortezomib, también conocido como PS-341, Velcade™, es un potente inhibidor de la actividad quimiotríptica del proteasoma 26S (Russo et al., 2007). El primer mecanismo de acción que se le atribuyó al bortezomib fue la inhibición de NFκB a través de la estabilización de IκB. Además de este efecto sobre NFκB, también provoca la estabilización de proteínas proapoptóticas como p53 o Bax, mientras que reduce los niveles de las antiapoptóticas como Bcl2 (Adams, 2004). Su eficacia ha sido probada en ensayos en fase II/III en pacientes con mieloma múltiple y linfomas malignos (Russo et al., 2007)

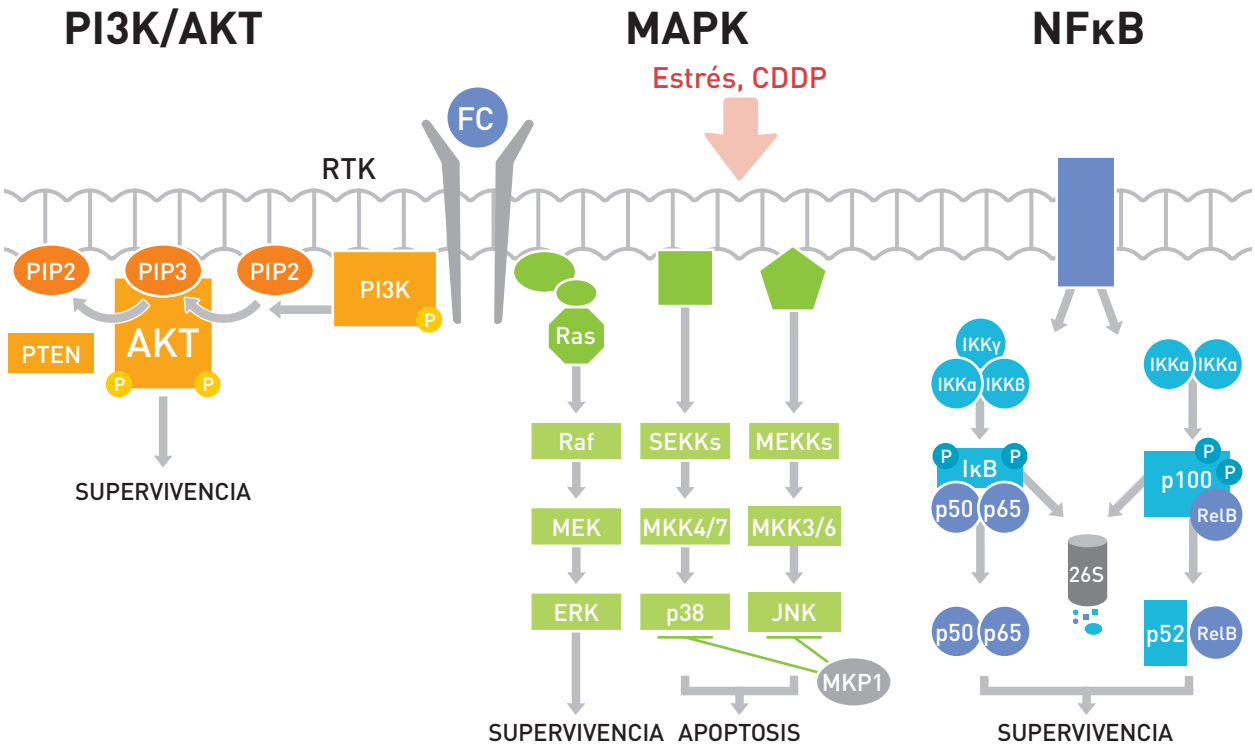

Figura 2. Esquema de las vías de señalización celular implicadas en la respuesta a CDDP abordadas en este trabajo. La unión de factores de crecimiento (FC) a receptores tirosina quinasa (cómo son IGFR y EGFR) pueden activar vías de señalización celular como la vía de PI3K/AKT o la vía de la MAPK ERK involucrada esencialmente en viabilidad celular. Por otra parte las señales de estrés, como por ejemplo el tratamiento con CDDP, provocan la activación de las MAPK, JNK y p38, con efectos normalmente anti-proliferativos y anti- apoptóticos. Las MAPK se encuentran reguladas de forma negativa por la fosfatasa MKP1, sobre todo JNK y p38. Finalmente el factor de transcripción NFκB, juega un papel importante en procesos de supervivencia celular a través de su activación por diferentes receptores (receptores de TNFα o receptores de tipo Toll) de las rutas canónica y no canónica dando lugar a la expresión de genes antiapoptóticos. Todas estas vías se han visto implicadas en la señalización celular en respuesta a CDDP.

4. BIOMARCADORES EN CÁNCER DE PULMÓN

Los biomarcadores en cáncer se definen como factores que pueden medirse de forma objetiva y que nos permiten distinguir entre procesos patológicos y normales y nos dan una idea de las características intrínsecas del tumor, como crecimiento y agresividad (biomarcadores de pronóstico), o que pueden predecir la respuesta a un fármaco (biomarcadores predictivos), aunque a veces la diferencia entre ambos tipos de biomarcadores es difusa. En algunos tipos de cáncer es difícil obtener un diagnóstico temprano, ya que los síntomas se desarrollan cuando

el tumor es muy grande, y los resultados que se obtienen tras el tratamiento son modestos. Por ello, encontrar herramientas que permitan un diagnóstico temprano es muy importante. Además, al ser el cáncer una enfermedad tan heterogénea, es fácil encontrar pacientes con un mismo tipo tumoral que responden de forma diferente al tratamiento (tanto en efectividad como en la citotoxicidad de los mismos), por tanto, lo ideal es poder disponer de una terapia personalizada para cada individuo en función de las características del tumor que presenten, para lo que es esencial identificar unos buenos biomarcadores.

Las características de un buen biomarcador son según la EDRN, red de investigación de la detección temprana que pertenece al NCI (Instituto Nacional de Investigación de Estados Unidos):

- Que sea capaz de distinguir individuos enfermos de sanos con un alto grado de exactitud
- Que se encuentre presente en los primeros estadios de la enfermedad
- Que sea fácilmente medible en fluidos corporales (suero/plasma, orina, esputo, saliva y lavado ductal)
- Que de lugar al desarrollo de un test de diagnóstico que finalmente tenga impacto en la tasa de mortalidad.

4.1 BIOMARCADORES y TIPOS TUMORALES

Hoy en día se utilizan biomarcadores en clínica para el uso de lo que se llaman terapias dirigidas, es decir, los biomarcadores son las dianas moleculares de las drogas que se van a utilizar, como por ejemplo, el trastuzumab (Herceptin, Roche/Genentech), aprobado para el tratamiento en cáncer de mama para individuos que tiene amplificación o sobreexpresión de HER2, el imatinib (Gleevec; Novartis), el cual está aprobado para leucemia mieloide crónica, y actúa inhibiendo al producto de la fusión de los genes BCR/ABL que está presente en estos pacientes. El vemurafenib (Zelboraf; Daiichi Sankyo/Roche) un inhibidor de la quinasa BRAF, aprobado por la FDA para pacientes de melanoma metastático con la mutación BRAFv600 en sus tumores. El crizotinib (Xalkori; Pfizer), un inhibidor de la quinasa ALK, aprobado para el tratamiento de pacientes de CNMP que tengan una reorganización del gen de *ALK (EML4-ALK).* Además también se utilizan inhibidores de EGFR en pacientes de CNMP que tengan amplificación o mutaciones en EGFR como el gefitinib (Iressa; AstraZeneca) o el erlotinib (Tarceva; Genentech).

En CNMP aparte de EGFR y EML4-ALK, se están estudiando otros marcadores, así como las posibles terapias dirigidas contra ellos, estando algunos en estas terapias en fase III de ensayos clínicos (Tabla 2). (Sudhindra et al., 2011)

BIOMARCADOR	PRONÓSTICO	PREDICTIVO
Subunidad de la reparación por escisión de nucleótidos 1 (ERCC1)	Estadios tempranos (operados): Altos niveles de ERCC1 ⟶ Bueno Bajos niveles de ERCC1 ⟶ Malo	Terapia basada en platinos: Altos niveles de ERCC1 ⟶ Resistencia Bajos niveles de ERCC1 ⟶ Sensibilidad
Ribonucleótido reductasa M1 (RRM1)	Estadios tempranos (operados): Altos niveles de RRM1 ⟶ Bueno Bajos niveles de RRM1 ⟶ Malo	Terapia basada en Gemcitabina : Altos niveles de ERCC1 ⟶ Resistencia Bajos niveles de ERCC1 ⟶ Sensibilidad
Receptor del factor de crecimiento epidérmico (EGFR)	Enfermedad avanzada: EGFR mutado ⟶ Bueno	Terapia basada en Erlotinib/Gefitinib: EGFR mutado ⟶ Buena respuesta
Susceptibilidad al cáncer de mama tipo 1 (BRCA1)	¿?	Terapia basada en platinos: Altos niveles de BRCA1 ⟶ Resistencia Terapia basada en Taxanos: Bajos niveles de BRCA1 ⟶ Sensibilidad
Timidilato sintetasa (TS)	¿?	Terapia basada en Pemetrexsed: Altos niveles de TS ⟶ Resistencia Bajos niveles de TS ⟶ Sensibilidad
ß-Tubulina	¿?	Terapia basada en Taxanos: Altos niveles de ß-Tubulina ⟶ Resistencia
Proteína 4 similar a la proteína asociada a microtúbulos de equinodermos-Quinasa del linfoma anaplásico (EML4-ALK)	¿?	Terapia basada en Erlotinib/Gefitinib: Mutación EML4/ALK ⟶ Resistencia Terapia basada en Crizotinib: Mutación EML4/ALK ⟶ Sensibilidad

Tabla 2. Listado de los biomarcadores descritos en CNMP así como su valor pronóstico y predictivo. Tabla adaptada de Sudhindra et al., 2011

4.2 GENES SILENCIADOS EPIGENÉTICAMENTE COMO BIOMARCADORES

La hipermetilación del ADN es un proceso temprano en cáncer, lo que podría proporcionar herramientas a los clínicos para un diagnóstico precoz. En los últimos años se han descrito una gran cantidad de genes silenciados por metilación de su promotor e implicados en progresión tumoral. Anteriormente se estudiaba el estado del promotor de un gen candidato, cuyos niveles de expresión estaban disminuidos en algún tipo de tumor en particular o en varios tipos tumorales. Sin embargo en los últimos años ha aumentado el número de estudios que se centran en la identificación global de genes hipermetilados, combinando la biotecnología de microarrays con la reactivación de genes silenciados epigenéticamente usando compuestos desmetilantes del ADN e inhibidores de las desacetilasas de histonas en diversas líneas celulares humanas, de diferentes tipos tumorales como vejiga (Liang et al., 2002), riñón (Ibanez de Caceres et al., 2006), colorrectal (Suzuki et al., 2002), esófago (Yamashita et al., 2002), próstata (Ibragimova et al., 2010) y pulmón (Shames et al., 2006). Por otra parte, también en los últimos años, se ha producido un avance en el desarrollo de técnicas de detección de ADN metilado, lo que ha permitido la identificación de cambios en los niveles y grado de metilación en fluidos corporales (sangre, orina, saliva, lavado bronquioalveolar, lavado ductal, deposiciones, etc.). Además, gracias a que el proceso de metilación del ADN es reversible mediante el uso de agentes desmetilantes del ADN e inhibibidores de desacetilasas de histonas; existe la posibilidad de la terapia epigenética en clínica (Egger et al., 2004), como el uso de decitabine (5-aza-deoxicitidina), o el ácido suberoilanilido hidroxámico (SAHA), principalmente usados en terapia de tumores líquidos como leucemias.

En cáncer de pulmón se han encontrado diferentes genes cuyos promotores se encuentran hipermetilados. Uno de los más importantes es *CDKN2A,* que codifica el inhibidor del ciclo celular p16, éste se encuentra silenciado en un 67% de los adenocarcinomas y en un 70% de los carcinomas escamosos. La metilación de este gen parece ser un evento temprano en cáncer de pulmón. Además, algunos genes supresores de tumores metilados en cáncer de pulmón, se empiezan a presentar como buenos marcadores predictivos de recurrencia temprana cuando se observa su presencia en ADN de ganglios linfáticos negativos en el análisis anatomopatológico de pacientes con cáncer de pulmón (Brock et al., 2008).

Sin embargo aún se desconocen genes silenciados epigenéticamente que se utilicen como biomarcadores predictivos de respuesta a platinos en CNMP.

OBJETIVOS

El objetivo principal de este trabajo es profundizar en el conocimiento de los mecanismos y vías de señalización implicadas en el proceso de resistencia a CDDP para identificar biomarcadores que predigan la respuesta a dicho quimioterápico en CNMP, para ello dividimos el trabajo en los siguientes objetivos parciales:

Objetivo1. Estudio de los cambios epigenéticos mediados por la exposición a CDDP en CNMP.

- Identificación de genes cuya expresión disminuya en resistencia debido a la metilación de novo de sus promotores.

- Valoración de la implicación de la metilación de dichos genes en el proceso de resistencia a CDDP y estudio de los mecanismos por los cuales esto ocurre.

- Correlación de los datos obtenidos a partir de los objetivos anteriores con los datos en muestras de pacientes de CNMP.

Objetivo 2. Estudio de la función de la fosfatasa MKP1 y de las vías de señalización PI3K/AKT y NFκB en la respuesta a CDDP en CNMP.

MATERIALES, MÉTODOS Y RESULTADOS

Capítulo 1

IGFBP-3 hypermethylation-derived deficiency mediates cisplatin resistance in non-small-cell lung cancer.

El tratamiento con CDDP puede inducir hipermetilación *de novo* del ADN, proceso que podría estar implicado en el desarrollo de resistencia a dicho fármaco por inactivación de genes requeridos para inducir el efecto citotóxico de la droga. En nuestro laboratorio se establecieron líneas celulares resistentes a CDDP a partir de las células tumorales de CNMP H460 y H23. Con este grupo de dos líneas pareadas sensibles/resistentes y mediante un análisis de microarrays de expresión se identificaron aquellos genes cuya expresión disminuía en las líneas resistentes comparada con la de las líneas sensibles y que se reexpresaban después de un tratamiento de reactivación epigenética. En los genes que seguían este criterio, se procedió a una validación tanto de la expresión genética como de su regulación epigenética, observándose que entre otros, el gen *IGFBP-3* se encontraba silenciado en resistencia y reexpresado después del tratamiento epigenético en las células H23S/R, fenómeno que también se observó en las células de cáncer de ovario 41S/R (cedidas por Dr. Lloyd R Kelland). Además, el promotor del gen *IGFBP-3* estaba metilado en las líneas resistentes H23R y 41R, lo que coincidía con los niveles disminuidos del ADNc del gen en estas líneas. Para comprobar que la metilación del promotor de *IGFBP-3* estaba implicada en la resistencia a CDDP, inhibimos su expresión en la línea celular 41S y observamos que la sensibilidad a CDDP disminuía presentando unos valores intermedios entre los de las líneas 41R y 41S, lo que indicaba que los niveles de expresión de *IGFBP-3* tenían un papel importante en la respuesta a CDDP. Después, se valoró la viabilidad celular y el estado de metilación del promotor de *IGFBP-3* en 23 líneas tumorales adicionales y se observó que de las células tumorales con menor sensibilidad a CDDP, un 75% presentaban el promotor de *IGFBP-3* metilado.

Finalmente, se quiso estudiar si la metilación del promotor de *IGFBP-3* era un evento frecuente en tumores primarios que correlacionase con la respuesta a CDDP. Para ello se utilizaron 36 muestras frescas de tumores en estadios I y II de CNMP, que se expusieron a varias concentraciones de CDDP para obtener las IC_{50} de cada una de ellas y de las cuales se estudió el estado de metilación del promotor de *IGFBP-3*. El análisis de estos datos agrupa a los pacientes diferencialmente en sensibles (17/36) o resistentes (19/36) a CDDP. Además se observó que el promotor de *IGFBP-3* se encontraba metilado con más frecuencia en las muestras resistentes a CDDP (74%) que en las sensibles (12%), y que la correlación entre el estado de metilación de *IGFBP-3* y la respuesta a CDDP era de un 81%. Finalmente se comparó el estado de metilación del promotor de *IGFBP-3* con la historia clínica de los pacientes y se observó que aquellos pacientes cuyas muestras no presentaban el promotor de *IGFBP-3* metilado tenían una supervivencia libre de enfermedad mayor que aquellos que si presentaban metilado el promotor. Con estos resultados podemos concluir que la pérdida de expresión de *IGFBP-3* por hipermetilación del promotor, da lugar a una reducción en la sensibilidad a CDDP en células tumorales de CNMP. Además, el estado de metilación de *IGFBP-3* antes del tratamiento podría utilizarse como un biomarcador en clínica para predecir el resultado de la quimioterapia, ayudando a identificar pacientes que podrían beneficiarse de la monoterapia con CDDP o en combinación con tratamientos de reactivación epigenética.

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ORIGINAL ARTICLE

IGFBP-3 hypermethylation-derived deficiency mediates cisplatin resistance in non-small-cell lung cancer

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Cisplatin-based chemotherapy is the paradigm of non-small-cell lung cancer (NSCLC) treatment; however, it also induces *de novo* DNA-hypermethylation, a process that may be involved in the development of drug-resistant phenotypes by inactivating genes required for drug-cytotoxicity. By using an expression microarray analysis, we aimed to identify those genes reactivated in a set of two cisplatin (CDDP) resistant and sensitive NSCLC cell lines after epigenetic treatment. Gene expression, promoter methylation and CDDP-chemoresponse were further analyzed in three matched sets of sensitive/resistant cell lines, 23 human cancer cell lines and 36 NSCLC specimens. Results revealed specific silencing by promoter hypermethylation of *IGFBP-3* in CDDP resistant cells, whereas *IGFBP-3* siRNA interference, induced resistance to CDDP in sensitive cells ($P<0.001$). In addition, we found a strong correlation between methylation status and CDDP response in tumor specimens ($P<0.001$). Thus, stage I patients, whose tumors harbor an unmethylated promoter, had a trend towards increased disease-free survival (DFS). We report that a loss of *IGFBP-3* expression, mediated by promoter-hypermethylation, results in a reduction of tumor cell sensitivity to cisplatin in NSCLC. Basal methylation status of *IGFBP-3* before treatment may be a clinical biomarker and a predictor of the chemotherapy outcome, helping to identify patients who are most likely to benefit from CDDP therapy alone or in combination with epigenetic treatment.
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Introduction

Non-small-cell lung cancer (NSCLC) comprises more than 80% of lung cancers, with 1.2 million new cases worldwide each year (WCR, 2003; Jemal *et al*., 2008). Cisplatin (CDDP)-based chemotherapy remains the standard treatment in combination with other chemotherapeutic agents; however, NSCLC patients frequently exhibit chemotherapy resistance. Molecular mechanisms of drug resistance remain unclear and are believed to be multifactorial, involving host factors, numerous molecular events and genetic and epigenetic changes (Gottesman, 2002). In addition, chemotherapeutics induce epigenetic changes in the promoter area of specific genes, altering expression and possibly underlying resistance in many tumor types (Baker *et al*., 2005).

The aberrant DNA hypermethylation of CpG islands in the gene promoter region is well-described as an alternative mechanism for tumor suppressor genes silencing in cancer cells. This includes lung cancer cells, in which MGMT, p16, RARb, TIMP-3 and DAPK have been reported as hypermethylated (Merlo *et al*., 1995; Esteller *et al*., 1999; Zochbauer-Muller *et al*., 2001). In addition, drug-induced DNA hypermethylation could be a mechanism of tumor cell response to chemotherapy agents (Nyce, 1997; Strathdee *et al*., 1999; Shen *et al*., 2004; Zhang *et al*., 2004). In both lung adenocarcinoma (HTB-54) and rhabdomyosarcoma human cells (CCl-136), pulse exposure to CDDP is associated with drug-induced DNA hypermethylation (Nyce, 1989), an event that has also been reported *in vivo* (Koul *et al*., 2004). Therefore, one possible reason for the development of chemoresistance in NSCLC might be the epigenetic inactivation of certain tumor suppressor gene as a consequence of chemotherapy treatment. This epigenetic silencing could be reverted by demethylating drugs and histone deacetylase inhibitors such as 5 Aza-2deoxycytidine (5Aza-dC) and Trichostatine A (TSA). Both drugs act in synergy by depleting methyltransferase activity (Baylin *et al*., 1998) and reversing the formation of transcriptionally repressive chromatin structure (Marks *et al*., 2004). Thus, the association between promoter-demethylation of *hMLH1* by

pharmacological treatment and reversal of drug-resistance has been reported in human tumor xenografts (Plumb *et al*., 2000; Steele *et al*., 2009).

Many epigenetically silenced tumor suppressor gene involved in cancer-chemoresistance likely remain to be identified as most studies have focused on a limited number of candidate genes (Strathdee *et al*., 1999; Shen *et al*., 2004). Therefore, a microarray-based screening pairing the differential genetic profile of sensitive and CDDP-resistant cell lines with an epigenetic reactivation approach has the advantage of a global analysis that should preferentially identify epigenetically silenced genes as a result of acquired CDDP-chemoresistance.

In this study, we first established two CDDP-resistant cell lines derived from the sensitive H23 and H460 NSCLC cell lines. Screening for genes differentially expressed in those resistant cells compared with their drug-sensitive parent cells and re-expressed after 5Aza-dC and TSA treatments is a new approach that can provide signatures for the identification of target genes for chemotherapy profiling. This process may lead to further elucidation of the biology of treatment response in NSCLC. We have identified a panel of genes showing this pattern, and found the *insulin-like growth factor-bindin protein-3* (*IGFBP-3*) gene specifically downregulated through promoter-hypermethylation in the resistant phenotypes.

The biological significance of *IGFBP-3* is of great importance in controlling cell growth, transformation and survival. It is the main IGFBP-family member, physiologically, *IGFBP-3* binds IGF-I with stronger affinity than the specific IGF-I receptor (IGFI-R) to the cell membrane, blocking their interaction and, therefore, the IGF-I mitogenic and anti-apoptotic actions. It is induced by wild-type p53 and interacts with the TGF-β and EGFR pathways (Buckbinder *et al*., 1995; Rajah *et al*., 1997; Guix *et al*., 2008). *IGFBP-3* has been recently reported by the authors and others as a candidate tumor suppressor gene under epigenetic regulation in several tumor types including lung, kidney and ovarian cancer (Chang *et al*., 2002b; Ibanez de Caceres *et al*., 2006; Wiley *et al*., 2006), and although its role in tumor progression has been studied, its function in acquired resistance to CDDP, chemotherapy response prediction as well as in drug-resistance phenotype identification remains to be characterized. In this study we examined the promoter methylation profile of *IGFBP-3* regarding sensitivity to CDDP in 27 human cancer cell lines and in a panel of 36 NSCLC specimens. We report that a loss of *IGFBP-3* expression mediated by aberrant promoter hypermethylation results in a reduction of tumor cell sensitivity to CDDP treatment in NSCLC.

Results

Establishment of NSCLC human cell lines resistant to CDDP

We established two human NSCLC cell lines resistant to CDDP, H23R and H460R, which were selected after a final exposure to 0.2 and 0.5 μg/ml CDDP respectively, showing approximately four times more drug resistance than the matched parental cell line (4.07 and 3.89 resistant index; $P<0.001$ and $P<0.002$) (Supplementary Figures 2a, b and c). Both cell lines showed a similar CDDP resistant-index to 41R ovarian cancer cells (4.8; $P<0.001$) (Supplementary Figure 2c).

Identification of genes induced by 5Aza-dC and TSA treatment in CDDP-resistant cell lines

We identified a group of genes under direct or indirect regulation of 5Aza-dC and TSA that were involved in important tumor-related pathways, including cell growth, apoptosis and negative regulation of cell-cycle progression. It is interesting to note that only three genes were commonly re-expressed in both cell lines at similar levels to the sensitive parental cells. We also selected a second group of six genes specifically re-expressed in each cell line, representing, as expected, significant inter-individual variation in drug response (Figure 1a).

For gene validation, we first tested their expression profile by RT–PCR, confirming the results from microarray analyses in all nine genes analyzed. The expression of *ARRDC-4*, *DKK1* and *DUSP5* was dramatically decreased in both H23R and H460R cell lines compared with their parental line, whereas a strong signal in H23R-T and H460R-T cells confirmed re-expression after 5Aza-dC and TSA treatment. *AREG, GDF-15* and *IGFBP-3* followed the same pattern in H23 cells, whereas *NNAT*, *PHLDA1* and *S100A2* did in H460 cells (Figure 1b). We previously reported that *GDF-15* was methylated in normal tissues (Ibanez de Caceres *et al*., 2006); therefore, we next used bisulfite sequencing (BS) to validate the promoter-methylation status in the remaining genes containing a real CpG island. Promoters of *ARRDC4*, *DKK1*, *DUSP5*, *AREG* and *PHLDA1* were unmethylated in all experimental groups, indicating that they are not under direct epigenetic regulation, as an example, the methylation status of *ARRDC4* is shown in Figure 1c. The *NNAT* gene was methylated in DNA from non-neoplastic lung, H460 and H460R cells, suggesting possible epigenetic regulation that could explain the intense signal in H460R cells after reactivation treatment. Data from *DKK1*, *DUSP5*, *AREG*, *PHLDA1* and *NNAT* are shown in Supplementary Figure 3.

IGFBP-3 CpG-island study and methylation analysis

For *IGFBP-3,* we studied a promoter CpG island located $-518/+744$ from the first exon, where we examined the 688 bp upstream from the ATG site. The island was unmethylated in non-neoplastic lung tissue DNA, whereas H23R resistant-cells were more densely methylated than H23S, in accordance with the decrease observed in gene expression. In addition, re-activation treatment restored unmethylated promoter and gene expression in H23R (Figure 2). We used the matched 41S and 41R cells to verify that *IGFBP-3*-methylation status was directly related with the CDDP-resistance process, as ovarian cancer is, like NSCLC, a tumor type that exhibits frequent resistance after CDDP-based

a

Gene ID	Gene Name	Gene Symbol	Cell Lines	Chromosome Location	CpG Island	ALUs	Methylation
NM_183376	Arrestin domain containing 4	*ARRDC4*	H23/H460	15q26.3	Y	N	U
NM_012242	Dickkopf homolog 1 (Xenopus laevis)	*DKK1*	H23/H460	10q11.2	Y	N	U
NM_004419	Dual specificity phosphatase 5	*DUSP5*	H23/H460	10q25	Y	N	U
NM_001657	Amphiregulin preproprotein	*AREG*	H23	4q13.3	Y	N	U
NM_004864	Growth differentiation factor 15	*GDF15*	H23	19p13.11	Y	N	U
NM_000598	Insulin-like growth factor binding protein 3	*IGFBP-3*	H23	7p13	Y	N	M
NM_181689	Neuronatin, transcript variant 2	*NNAT*	H460	20q11.23	Y	N	U
NM_007350	Apoptosis-associated nuclear protein PHLDA1 mRNA	*PHLDA1*	H460	12q21.2	Y	N	U
NM_005978	S100 calcium binding protein A2	*S100A2*	H460	1q21.3	N	-	-

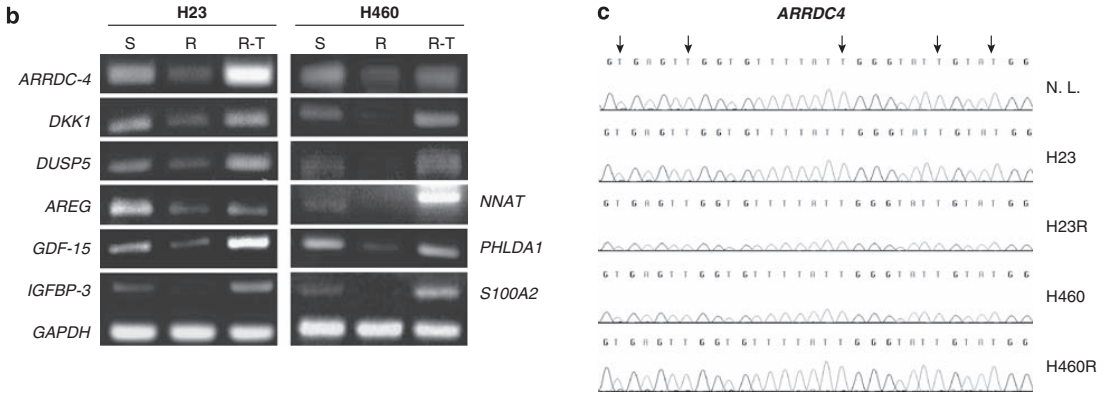


Figure 1 Identification of resistant genes under epigenetic regulation. (**a**) Selected genes meeting the described criteria, in both resistant cell lines (H23/H460) or in each cell line independently (H23 or H460). (**b**) RT–PCR in parental (S), resistant (R) and resistant cell lines under epigenetic reactivation (R-T). (**c**) Bisulfite sequencing of *ARRDC4* as an example of an unmethylated gene, indicated by the presence of T instead of C in the positions indicated by the arrows in all experimental groups: non-neoplastic lung tissue (NL), H23, H23R, H460 and H460R cell lines.

therapy. Supporting our results, we found the *IGFBP-3* promoter completely unmethylated in 41S cells and densely methylated in 41R cells, whereas 5-Aza-dC/TSA treatment again restored promoter unmethylation and gene expression, confirming that *IGFBP-3*-hypermethylation occurs in those cells as a CDDP resistance-specific process. Furthermore, using qRT–PCR, we confirmed that *IGFBP-3* expression was 1000-fold higher in 41S cells than in H23S cells, in which the *IGFBP-3* promoter is semi-methylated (Supplementary Figure 4). Accordingly, we established that *IGFBP-3*-methylation in 41R cells parallels a decrease in gene expression of more than 10 000-fold compared with 41S cells (Supplementary Figure 4), whereas reactivation treatment increased the expression by 10 times. These results indicate that hypermethylation of *IGFBP-3* is specific to CDDP-resistant cell lines.

IGFBP-3-promoter methylation mediates the response to CDDP

To investigate if *IGFBP-3*-promoter hypermethylation mediates chemosensitivity to CDDP or is a consequence of treatment, we silenced expression in 41S cells, as in these cells *IGFBP-3* is highly expressed and therefore susceptible to being successfully knocked-down. Transfection of the siRNA-negative-control did not affect the *IGFBP-3* expression pattern in 41R and 41S cells (Figure 3a). *IGFBP-3* expression was successfully silenced in 41S cells with 75% interference efficiency 24 h after transfection, effect that was maintained after 72 h with an approximately 70% efficiency compared with 41S cells transfected with control-RNAi (Figure 3b). This *IGFBP-3* silencing induced a decrease in CDDP sensitivity compared with cells transfected with the negative control, resulting in a significant increase in the IC_{50} value in response to CDDP (0.5 μg/ml vs 0.3 μg/ml, $P<0.001$). Those cells showed an intermediate sensitivity degree between 41S and 41R cells transfected with the negative control, with a resistance index lower than 41R RNAi NC cells (1.67 vs 4.1, $P<0.001$), in which *IGFBP-3* is completely silenced by promoter methylation (Figures 3c and d). These results confirm *IGFBP-3* promoter hypermethylation as an event that mediates the response to CDDP.

Next, CDDP viability response and *IGFBP-3* methylation status were measured in 23 additional cancer cell

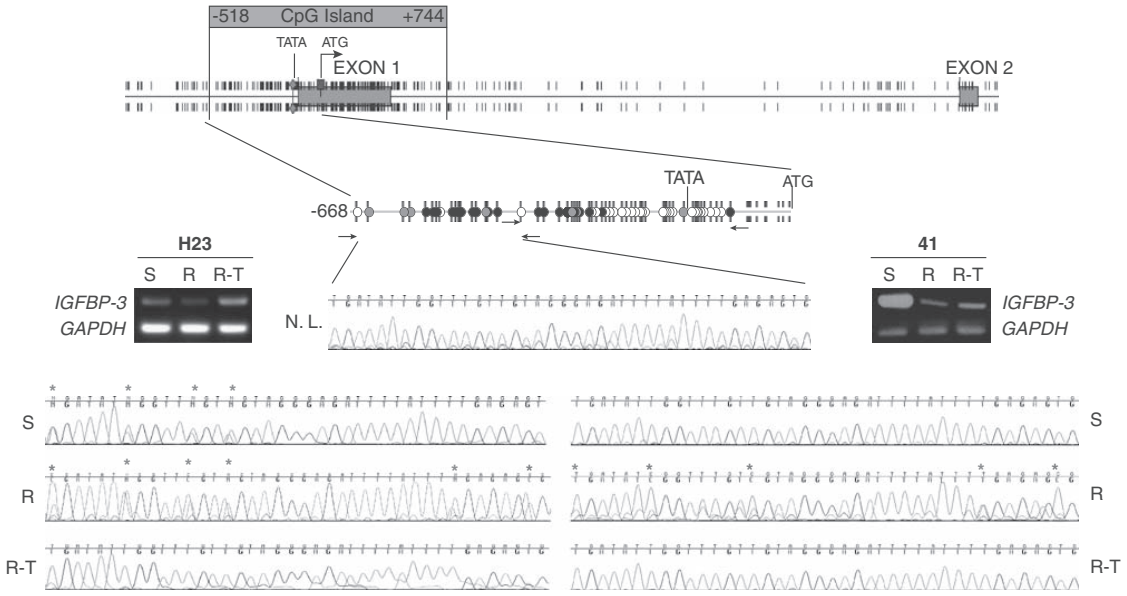


Figure 2 Bisulfite sequencing of the *IGFBP-3* gene promoter. Analysis of the CpG island from non-neoplastic lung tissue DNA (NL) and two sets of CDDP sensitive (S), resistant (R) and resistant-treated (R-T) cell lines derived from H23 (left set) and 41 (right set) cell lines. The CpG island location is indicated by the red square and covers the first exon. The area analyzed is that indicated between the two pairs of arrows at the top of the figure. Circles represent the methylation frequency of each CpG site analyzed from H23R and 41R cell lines; white: never or low methylation frequency (0–15%); gray: medium methylation frequency (15–30%) and black: high methylation frequency (>30%). The presence of C preceding a G in the sites indicated by the red asterisks demonstrates that these cytosines were methylated, whereas the blue ones indicate semi-methylated positions. The presence of T instead of C in the same positions in NL and in the R-T cell lines indicates unmethylated positions in no-tumor DNA and after epigenetic-reactivation treatment. RT–PCR of both sets linking the cell lines methylation profile is shown at the top of each set. A full colour version of this figure is available at the *Oncogene* journal online.

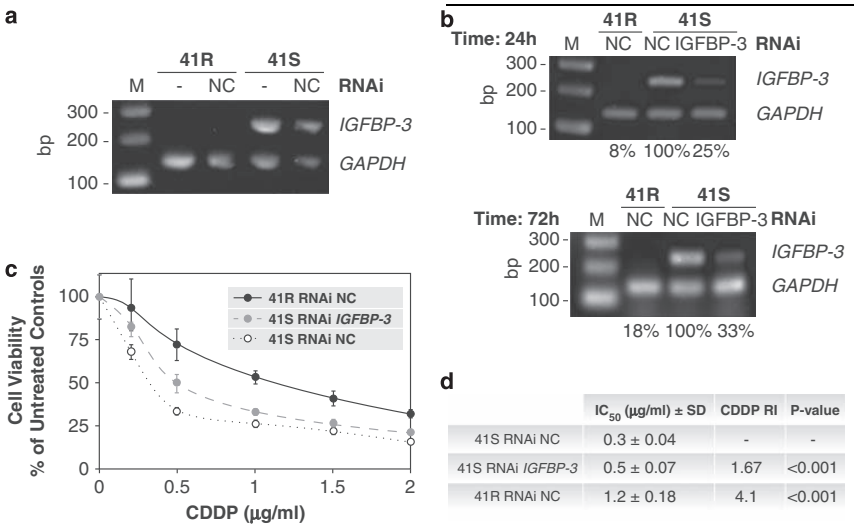
lines, in order to study the correlation between promoter hypermethylation and CDDP-resistance, and to confirm the most frequent methylation positions for the methylation-specific PCR (MSP) primer design (Supplementary Table 1). Results showed different viability responses to CDDP independently of tumor type. In all, 16 out of 23 (70%) cell lines showed low CDDP-sensitivity ($IC_{50} > 2.5\,\mu g/ml$), with *IGFBP-3* methylated in 12 (75%), probably as a consequence of metastasic origin of cell lines (Supplementary Figure 1). The MSP primers designed by comparing the BS from the cell lines were used to analyze the *IGFBP-3*-methylation status in DNA from the formalin-fixed, paraffin-embedded-NSCLC tumors, as described below.

Specific methylation of IGFBP-3 in CDDP-resistant NSCLC primary tumors

To determine whether *IGFBP-3* methylation is a frequent event in primary tumors that correlates with the response to CDDP, we exposed 36 stage I/II NSCLC fresh tumors to various CDDP concentrations, analyzed their *IGFBP-3*-methylation status by MSP and studied their correlation with clinical history. All baseline characteristics vs CDDP-sensitivity and methylation status are summarized in Table 1, whereas individual patient data are listed in Supplementary Table 2.

According to receiver operating characteristic methodology, the IC_{50} index clearly delineated two groups of patients in terms of the sensitive or resistant profile to CDDP. The cut-off point was established on an IC_{50} of $7.5\,\mu g/ml$. Under these conditions, 19 out of the 36 samples were above this cut-off, and therefore, considered to have low sensitivity to CDDP, whereas 17 samples out of 36 were under this cut-off and considered highly sensitive. *IGFBP-3* was more frequently methylated in resistant (14 out of 19, 74%) than in sensitive tumors (2 out of 17, 12%) ($P < 0.001$); in addition, *IGFBP-3* was unmethylated in 15 out of the 17 (88%) highly sensitive samples (Figure 4c and Table 1). Consequently, we found an 81% (29 out of 36 samples) correlation between *IGFBP-3*-methylation status and response to CDDP (Figure 4c). In addition, we did not detect any CpG-site methylation in ten additional non-neoplastic lung samples. DNA extracted from three fresh samples was used to test *IGFBP-3*-methylation as an internal control for the MSP analysis (Figure 4b). An MSP sample is shown in Figure 4a; patients 22 and 34 are unmethylated, whereas 19, 6 and 30 are methylated, linking with the BS shown in Figure 4b.

When comparing data from pooled patients according to stage and methylation status of the *IGFBP-3*-promoter, we observed that patients diagnosed with pathological stage I, whose tumors harbor an unmethylated promoter, had a clearly increased DFS compared with methylated stage I patients. In addition, a clear trend of unmethylated stage I patients towards higher



d

	IC_{50} (µg/ml) ± SD	CDDP RI	P-value
41S RNAi NC	0.3 ± 0.04	-	-
41S RNAi *IGFBP-3*	0.5 ± 0.07	1.67	<0.001
41R RNAi NC	1.2 ± 0.18	4.1	<0.001

Figure 3 Effect of *IGFBP-3* silencing on sensitivity to CDDP. (**a**) *IGFBP-3* expression after transfection with siRNA negative control (NC) in 41S and 41R cells (**b**) *IGFBP-3* mRNA levels in 41R and 41S cells transfected with siRNA against *IGFBP-3* or NC at 24 and 72 h after transfection. cDNA quantity was estimated by PCR amplification of the *GAPDH* gene using ImageJ 1.37V N (http://rsb.info.nih.gov/ij/) (**c**) Viability curves of 41S cells transfected with siRNA against *IGFBP-3* (41S RNAi *IGFBP-3*) or with the negative control (41S RNAi NC) and 41R cells transfected with the negative control (41R RNAi NC), to CDDP at six different test-drug concentrations. Data were normalized to each untreated control, set to 100%. Data represent the mean ± s.d. of at least three independent experiments carried out with eight wells at each drug concentration for every cell line analyzed. (**d**) Data represent the IC_{50}, inhibitory concentration that kills 50% of the cell population 48 h after CDDP exposure; the CDDP-RI, or resistance index to CDDP was calculated as (IC_{50} from 41S RNAi *IGFBP-3* or 41R RNAi NC)/(IC_{50} 41S RNAi *IGFBP-3*) ± the s.d. $P<0.01$ was considered a significant change in drug sensitivity (Student's *t*-test). M, marker; bp, base pair.

Table 1 Baseline characteristics of included patients. Correlation between methylation status, resistance to cisplatin and clinical/pathological characteristics are shown

	N *(%)*	*Methylated*	*Unmethylated*	P	*Sensitive*	*Resistant*	P
Stage							
Stage I	22 (61.1)	12	10	0.172	7	15	0.043
Stage II	13 (36.1)	4	9		9	4	
Stage unknown	1 (2.8)	0	1		1	0	
TNM							
T1	9 (25)	6	3	0.288	0	9	0.005
T2	22 (61.1)	9	13		13	9	
T3	4 (11.1)	1	3		3	1	
T unknown	1 (2.8)	0	1		1	0	
N0	26 (72.2)	13	13	0.460	10	16	0.245
N1	9 (25)	3	6		6	3	
N unknown	1 (2.8)	0	1		1	0	
Histology							
Epidermoid	22 (61.1)	9	13	0.427	11	11	0.64
Adenocarcinoma	10 (27.8)	4	6		5	5	
Large cell	4 (11.1)	3	1		1	3	
In vitro resistance to cisplatin							
Resistant	19 (52.8)	14	5	<0.001			
Sensitive	17 (47.2)	2	15				

survival was evident in all strata (Figure 5b), but without statistical significance, mainly because of a small sample size (Figure 5a). Surprisingly, for stage II patients we observed the opposite scenario: patients that harbor an unmethylated promoter had a lower DFS than those methylated within the same stage.

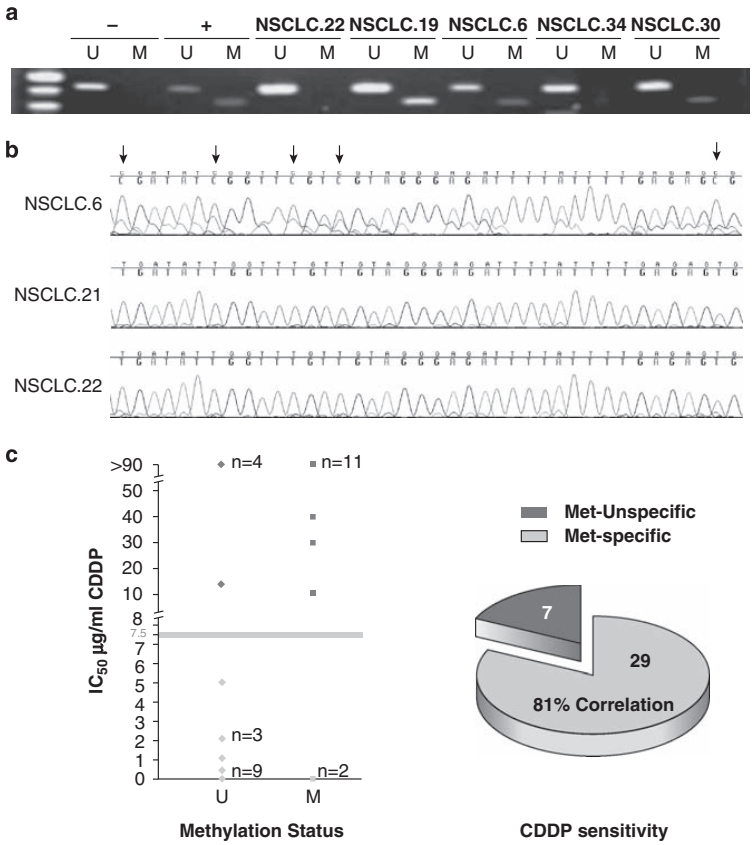


Figure 4 MSP of *IGFBP-3* in primary NSCLC tumors. (**a**) MSP example in the analyzed tumor samples: Presence of a PCR product in the methylated lane (M) indicates methylated alleles for *IGFBP-3*. PCR product in the unmethylated lane (U) from tumor DNA most likely arises from normal cell contamination of the tumor specimen. Tumor cell lines 41R and H1299 and primary tumors 5 and 15 were used indistinctly as positive controls, whereas cell line 41S and primary tumors 21 y 29 as negative controls. (**b**) BS of three primary tumors confirms MSP primer specificity. Arrows point to the methylated cytosines in sample 6. (**c**) The left figure represents CDDP sensitivity from the 36 NSCLC samples using the IC_{50} value against their methylation status. Samples with $IC_{50} \geqslant 7.5\,\mu g/ml$ are considered resistant to CDDP. The right panel represents the correlation between methylation status and CDDP sensitivity. Met-specific represents samples whose response to CDDP correlates with *IGFBP-3* methylation status, whereas Met-unspecific represents samples with no correlation.

Discussion

A central issue to better understanding the natural history of NSCLC is why disease becomes refractory to CDDP-based chemotherapy treatment. Cisplatin has extensive antitumoral activity, whereas its toxicity in normal tissues is relatively restricted to specific cellular types such as gastrointestinal enterochromaffin cells, kidney convoluted tubules or dorsal root ganglia (McKeage, 1995), suggesting an epigenetic influence in toxicity. In addition, CDDP exposure is associated with drug-induced DNA hypermethylation *in vitro* (Nyce, 1989) and clinically (Koul *et al*., 2004), indicating the importance of epigenetic changes in the rapid induction of resistance.

Most studies involving epigenetic chemotherapy-acquired resistance have focused on a limited number of candidate genes, such as *p16* (Katsaros *et al*., 2004), *RASSF1A* (Yoon *et al*., 2001; Makarla *et al*., 2005) or *hMLH1* (Strathdee *et al*., 1999), which possibly contribute to resistance in ovarian, lung and other cancers. Here, we developed a microarray-based strategy to identify new potential targets of promoter hypermethylation in CDDP-resistant phenotypes in lung cancer, which are therefore potentially involved in the development of resistance. We established the cell lines H23R and H460R, with a CDDP-resistant index in accordance with the previously established cancer cell line 41R, assuming that similar resistant events could follow. NSCLC-resistant cell lines underwent epigenetic treatment, and were then compared with the parental and resistant cell lines through microarray assays. The ontology analysis identified a set of genes involved in several tumor progression pathways, similarly to a recently published genomic approach that identified apoptosis as one of the most represented

a

Stage and Methylation status		Stage I-Methylated	Stage I-Unmethylated	Stage II-Methylated	Stage II-Unmethylated
		Sig.	Sig.	Sig.	Sig.
Log Rank (Mantel-Cox)	Stage I Methylated		0.428	0.780	0.086
	Stage I Unmethylated	0.428		0.327	0.043
	Stage II Methylated	0.780	0.327		0.466
	Stage II Unmethylated	0.086	0.043	0.466	

b

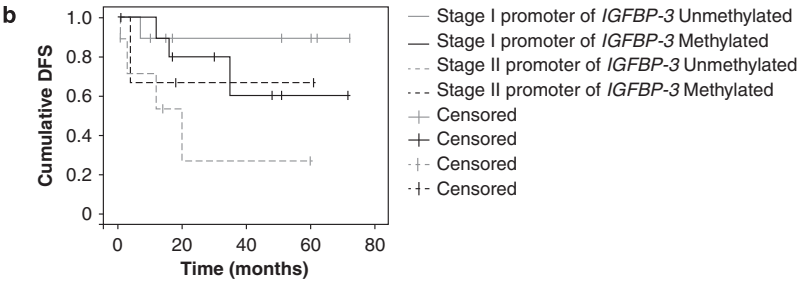


Figure 5 Pair comparison and Kaplan–Meier. Comparison between stages, *IGFBP-3* methylation status and cumulative disease-free survival in 36 patients diagnosed with stage I and II NSCLC.

pathways associated with CDDP-resistance (Riedel *et al*., 2008).

From the nine genes selected for further validation, three were commonly re-expressed in both resistant cell lines, whereas six other genes were specific for each cell line, indicating that CDDP and 5Aza-dC/TSA treatments affect different groups of genes and/or biological pathways, which could explain the high inter-individual variation in drug response described in lung cancer patients (Herbst *et al*., 2008). Further validation confirmed that all genes had basal expression in the parental cells, which highlights their importance in maintaining the sensitive phenotype, as they lacked or strongly diminished their expression in resistance. Confirming the relevance of our approach to identifying 'resistance-inductor-genes', it is interesting to note that the decreased expression of *DKK1* has been recently associated with acquired CDDP resistance in head and neck cancer (Gosepath *et al*., 2008). Epigenetic validation revealed that only one gene from our panel, *IGFBP-3*, had specific promoter methylation in CDDP-resistance, thereby indicating that the remaining genes are not under direct epigenetic regulation although their re-expression after 5Aza-dC/TSA treatment confirms recent findings (Morris *et al*., 2008). Those genes could be activated by an upstream regulatory gene or transcription factor whose expression was reactivated by demethylation, as is the reported case of gene *TGM2*, which is re-expressed in kidney cancer but epigenetically regulated upstream by *RASSF1A* (Ibanez de Caceres *et al*., 2006).

We focused on the epigenetic regulation of *IGFBP-3*. Our results show *IGFBP-3* strongly silenced by promoter hypermethylation in 41R and H23R-resistant cells in comparison with respective parental cells, with marked *IGFBP-3*-basal expression. In addition, demethylation treatment restored the unmethylated CpG positions as well as gene expression, thus confirming the epigenetic regulation of *IGFBP-3* in CDDP-resistant cell lines. Moreover, *IGFBP-3* was more highly expressed in the unmethylated cell line 41S than in H23 cells, which *IGFBP-3*-promoter is semi-methylated. Those results confirm the correlation between the decrease in *IGFBP-3* expression regulated by promoter hypermethylation and the loss of CDDP sensitivity. Furthermore, we here report that the *IGFBP-3*-promoter hypermethylation event is responsible for and not a consequence of CDDP-acquired resistance. This is because silencing of *IGFBP-3* expression in the 41S cells induced a strong decrease in CDDP sensitivity with a relative resistance index transitional to resistant 41R and sensitive 41S cells. We also tested the specificity of aberrant *IGFBP-3*-promoter methylation to detect CDDP sensitivity in primary tumors, and found that most of CpG dinucleotides were methylated in resistant but not in sensitive primary samples, indicating a significant correlation between *IGFBP-3*-methylation and CDDP chemosensitive-response. *IGFBP-3* was methylated in some patients before clinical exposure to CDDP, suggesting a possible intrinsic resistance to the drug. In fact, a follow-up study of those patients after chemotherapy treatment showed that patients diagnosed with pathological stage I and harboring an unmethylated *IGFBP-3* promoter had a clear trend towards higher survival, evident in all strata, compared with other groups. Our sample size was not large enough to demonstrate a statistically significant difference between all groups, but it is a starting point for future studies. The surprising results obtained from stage II patients are also size-limited, but it is possible that adjuvant therapy typically administered for stage II patients could have a role in observed differences within this stage. Unfortunately, we have no full data about adjuvant therapy administered in our set of patients.

From our results, we report that *IGFBP-3*-methylation could be a master key used for cell tumor

progression in conjunction with its biological functions. Induction of *IGFBP-3* gene expression by wild-type p53 is associated with enhanced secretion of an active form of *IGFBP-3* capable of inhibiting mitogenic signaling by the IGF-I (Buckbinder *et al*., 1995). The CGH profile of cell lines 41S and 41R indicate no changes in chromosome 17, whereas *p53* is located at (17p13) (Leyland-Jones *et al*., 1999), indicating that *IGFBP-3* promoter methylation at the *p53* regulatory element could cause gene silencing resistant to p53 in those cell lines. The IGF-I signaling pathway could mediate chemotherapy resistance of NSCLC cells through the activation of Akt/mTOR-mediated synthesis of survival proteins, thus protecting NSCLC cells from apoptosis induced by several drugs, as reported previously with drugs that inhibit tyrosine-kinase receptors including EGFR or IGFIR (Morgillo *et al*., 2006, 2007). Those authors suggest that resistance is caused by the activation of alternative cell survival signaling mechanisms, because those drugs do not inhibit proliferation at doses sufficient to suppress EGFR activation. In fact, we observed decreased expression of *IGFBP-3* in chemoresistant cell lines, therefore a higher amount of IGF-I should be available to join its own receptor and strongly activate the IGFIR pathway, inducing survival and maintaining proliferation in CDDP-resistant cells. In addition, in a p53 and/or IGF-I-independent pathway, methylation could also be the mechanism responsible for inhibiting the apoptosis mediated by *IGFBP-3* through the activation of the TGF-β receptor TβR-V, (Rajah *et al*., 1997; Huang and Huang, 2005).

IGFBP-3-methylation has been correlated with clinicopathological features indicative of poor prognosis in prostate and ovarian cancers (Wiley *et al*., 2006; Perry *et al*., 2007) and in the early stages of NSCLC patients (Chang *et al*., 2002a, 2002b), likely indicating that those patients have intrinsic resistance to CDDP. However, we know that those patients with unmethylated *IGFBP-3* in early stages could also progress towards a methylated promoter after CDDP treatment, thus showing acquired resistance. A follow-up study after chemotherapy treatment measuring aberrant *IGFBP-3* methylation in tumor DNA circulating in body fluids such as blood, bronchioalveolar fluid or saliva could identify this situation when CDDP-resistant cells arise, providing a non-invasive test to predict chemotherapy resistance.

In this study, we report that *IGFBP-3* has an inverse effect on the risk of NSCLC CDDP chemoresistance development. CDDP induces changes in *IGFBP-3* expression mediated by the acquisition of promoter hypermethylation, which promotes resistance to CDDP through various biological pathways. Therefore, basal methylation status of the *IGFBP-3* promoter before chemotherapy treatment may be a clinical biomarker predictor of the chemotherapy outcome of NSCLC patients, identifying those who are most likely to benefit from CDDP therapy. These results represent a new application of epigenetic cell control in chemotherapy resistance. This application involves the possible use of new highly sensitive and non-invasive tests based on the detection of aberrant methylation in tumor-circulating DNA (Carvalho *et al*., 2008), as well as the use of agents that reverse epigenetic changes. These agents have shown promising results in a mouse model of lung carcinogenesis and are being tested in lung cancer patients (Belinsky *et al*., 2003)

Materials and methods

Cell culture and viability to CDDP

A total of 23 human cancer cell lines (Supplementary Figure 1), representing nine different cancer types, were purchased from the ATCC (Manassas, VA, USA) or the ECACC (Sigma-Aldrich, Madrid, Spain) and cultured as recommended. The CDDP-resistant variants H23R and H460R were established following the methodology described previously (Plasencia *et al*., 2006) and further detailed in Supplementary Materials and Methods (Roninson, 2003; Chattopadhyay *et al*., 2006; Levina *et al*., 2008). To validate the results obtained from the resistant cell lines established in our laboratory, we also used CDDP-sensitive and resistant ovarian cancer cell lines 41M and 41MR, hereafter called 41S and 41R respectively, kindly provided by Dr Lloyd R Kelland (UK), and maintained in DMEM supplemented with 10% FBS.

NSCLC clinical samples and data collection

Fresh and formalin-fixed, paraffin-embedded surgical specimens were obtained from 36 patients who had undergone a complete resection (R0) for a histologically confirmed, early NSCLC. All patients had both a perioperative PET-CT scan showing localized disease and a pathological confirmation of stage I/II. In addition, an age of 18 years or older, intraoperative mediastinal-node dissection for reliable mediastinal staging or biopsy of nodes at N3 without any evidence of disease was inclusion criteria. Stage III, any involvement of tracheobronchial angle nodes (station 10), mixed histological features and previous diagnosis of cancer within the last 5 years were exclusion criteria. Histological slides obtained from each block were reviewed by an expert pathologist (M Nistal) to confirm the diagnosis and to guarantee at least 90% tumoral content. A total of 10 samples obtained from pulmonary biopsies with non-neoplastic lung pathology were used as control tissues. Follow-up was carried out according to the criteria used in the Medical Oncology Division from the University Hospitals La Paz and 12 de Octubre, including clinical assessments and thorax CT every 3 months for 2 years and every 6 months thereafter. Clinical, pathological and radiological data were recorded by an independent observer at the H. La Paz and blinded for statistical analysis.

5Aza-2dC and TSA treatment

5Aza-dC and TSA (Sigma-Aldrich) were stored as 5 mM and 330 μM stock solutions, respectively. For re-expression studies, resistant cell lines H23R, H460R and 41R were split to low density and exposed to 5Aza-dC and TSA or to PBS and ethanol (mock cells) as described previously (Ibanez de Caceres *et al*., 2006).

Oligonucleotide array hybridization and gene selection

Parental H-23 and H-460 cell lines, derived CDDP-resistant cell lines and resistant cell lines treated with 5Aza-dC and TSA were used for the oligonucleotide array hybridization. Total RNA was isolated and purified as described (Ibanez de Caceres *et al*., 2006) and used for the microarray and RT–PCR analysis. The microarray assay was carried out using the

Agilent gene expression platform 4X44 Whole Human Genome Oligo Microarray Kit, representing the 41 000 known genes and transcripts in the human genome. Sample amplification, labeling and scanning procedures followed the Agilent microarray protocol and are further described in Supplementary Materials and Methods.

Reverse transcription and qRT–PCR
Semiquantitative and real-time RT–PCR assays were carried out in all the experimental groups. In all, 5 μg of total RNA was reverse transcribed using oligo (dT)24 primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For quantitative real-time RT–PCR analysis, the amount of 1 μg of total RNA was retrotranscribed by High-Capacity cDNA Archive Kit (Applied Biosystems, Madrid, Spain). Next, each cDNA sample was analyzed in triplicate using the ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Madrid, Spain). Real-time PCR was carried out using Taqman Universal PCR Master Mix (Applied Biosystems, Madrid, Spain), containing ROX to normalize emissions. Primers and probe for *IGFBP-3* expression analysis were purchased from Applied Biosystems (Assay ID: Hs00181211_m1). Relative gene expression quantification was calculated according to the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) using GADPH and 18S rRNA as endogenous control genes and the H23S and 41S cell lines as calibrators. Normalized expression values were determined as follows: $10^{-(\Delta Ct\ \text{sample}-\Delta Ct\ \text{calibrator})}$, where ΔCt values were calculated by subtracting the Ct value of the target gene from the value of the mean between endogenous control genes. PCR settings and primer sequences are listed in Supplementary Materials and Methods and Supplementary Table 1.

siRNA transfection
The 41S and 41R cell lines were transfected with 150 nM *IGFBP-3* siRNA or negative control siRNA (HSS105266, Stealth Select RNAi and 2935–200, stealth RNAi Negative Control, Invitrogen, Barcelona, Spain) according to manufacturer directions. For the viability assay, cells were seeded in 24-well dishes at 50 000 cells per well 24 h after transfection with *IGFBP-3* siRNA or siRNA control, then treated with six different doses of CDDP for an additional 48 h and stained following the method described previously (Chattopadhyay *et al*., 2006). Cell viability was estimated relative to the density recorded over the same experimental group without drug exposure at same period of time (24 h following seeding of cells). Simultaneously, same experimental groups were seeded at 500 000 cells per plate and harvested at same incubation periods, 24 and 72 h after siRNA transfection, as a time-course to test by RT–PCR the preservation of siRNA effects on *IGFBP-3* expression.

BS and methylation-specific PCR
DNA from human cancer cell lines, NSCLC primary specimens and non-neoplastic lung tissues were isolated, bisulfite modified and then used for BS and MSP analysis as described (Ibanez de Caceres *et al*., 2006). PCR settings and primer sequences for BS and MSP are listed in Supplementary Materials and Methods and Supplementary Table 1.

Culture and CDDP treatment of human cancer tissues
To measure the sensitivity of the 36 primary NSCLC samples to CDDP, fresh tumors were minced, passed through a nylon mesh and enzyme disaggregated with collagenase type II and hyaluronidase in Dulbeco's modified Eagle's medium nutrient mixture F-12/Ham media (Sigma-Aldrich) with antibiotics. Cells were resuspended in 96-well microtiter plates followed by exposure to various concentrations of CDDP as described in Supplementary Materials and Methods.

Statistical analysis
Differentially expressed genes from the microarray analysis were selected using as a statistical method the *t*-test unpaired algorithm with Benjamini Hochberg as the FDR correction method for multiple testing corrections. Statistically significant ($P<0.05$ as adjusted *P*-value) genes were selected. Calculations were carried out using the gene expression analysis software GeneSpring (further details in *Oligonucleotide array hybridization and gene selection* from Supplementary Materials and Methods).

Receiver operating characteristic curves from NSCLC patients were obtained to identify the relation between sensitivity and resistance rate at a specific cut-off value established according to the best combination of sensitivity and false-positive rate (1–specificity) (1;0).

The Kaplan–Meier method was used to plot cumulative disease-free survival curves for patients diagnosed with stage I and unmethylated IGFBP-3 promoter, stage I methylated, stage II unmethylated and stage II methylated. Differences were compared with the log-rank method for every stratum. For patients without any evidence of relapse at the time of analysis, data on disease-free survival were censored at the time of last contact. Disease-free survival was defined as the time from surgery to clinical, radiological or histological evidence of relapse. Discrete variables (histology, T, N, stage, gender and methylation status at the IGFBP3 promoter and *in vitro* sensitivity/resistance to CDDP) were compared with the χ^2 test and corrections with Fisher's exact test were made when needed. Statistical significance was defined as $P<0.05$. Statistical analyses were done by using the SPSS software (version 17.0).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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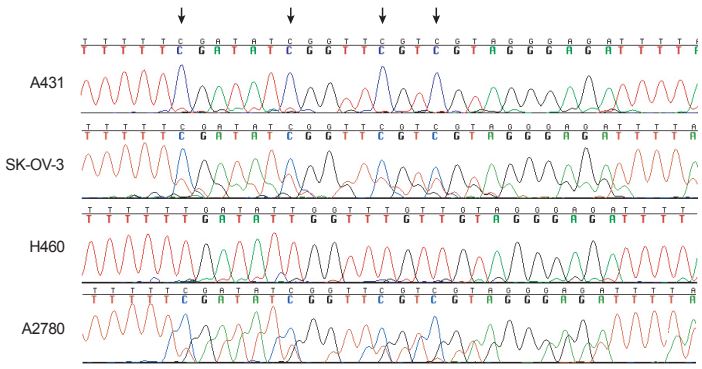
Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Material suplementario

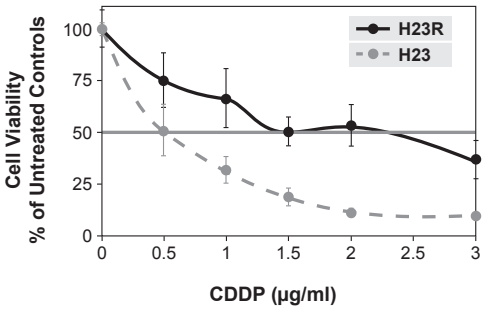
Human Cancer Cell line	Source and tumor type	IC_{50} (µg/ml)	Methylation
PC-3	Prostate, adenocarcinoma	8	U
BT474	Breast, ductal carcinoma	25	U
HT-29	Colon, colorectal adenocarcinoma	25	M
H727	Lung, bronchus carcinoid	9	M
H1299	Lung, carcinoma	6	M
OVCAR1	Ovary, adenocarcinoma	5	U
HeLa	Cervix, adenocarcinoma	3	M
TCCSUP	Bladder, transitional cell carcinoma	7	U
A-431	Skin, epidermoid carcinoma	2.5	M
RWP1	Pancreas, adenocarcinoma	5	M
PANC-1	Pancreas, epithelioid carcinoma	4	M
IMIM-PC-2	Pancreas, adenocarcinoma	3	M
SW 780	Bladder, transitional cell carcinoma	2.5	M
LNCaP	Prostate, carcinoma	2.5	M
SK-OV-3	Ovary, adenocarcinoma	3	M
OVCAR-3	Ovary, adenocarcinoma	3	M
LoVo	Colon, colorectal adenocarcinoma	2	M
A549	Lung, carcinoma	2	U
H23	Lung, adenocarcinoma	0.52	M
UM-UC-3	Bladder, transitional cell carcinoma	0.7	M
H 460	Lung, carcinoma	0.29	U
SK-BR-3	Breast, adenocarcinoma	0.7	U
A2780	Ovary, carcinoma	0.25	M



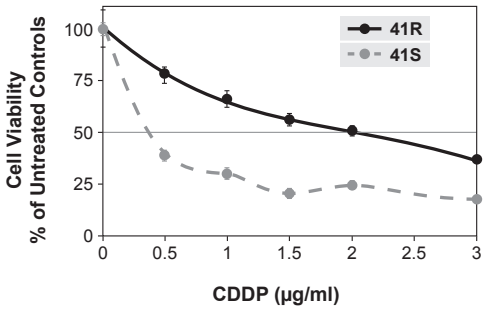
Supp. Figure 1

Supplementary Figure 1: *IGFBP-3* Methylation status and cell viability response to CDDP in a panel of 23 cancer cell lines representing nine different human cancer types. The upper panel shows the name of each cell line analyzed, the source and tumor type, the cell viability response to CDDP represented by the IC_{50} and the methylation status. U represents the unmethylated status while the methylated is represented by M. The lower panel shows the bisulfite sequencing of four cell lines from the upper panel. The arrows point the CpG positions susceptible of methylation. Cell lines A431, SK-OV-3 and A2780 are methylated while in H460, T instead of C, indicate that is unmethylated. The MSP primers used to analyze the *IGFBP-3* methylation status in DNA from NSCLC primary tumors, were designed by comparing the bisulfite sequences from the 23 cell lines, and contained the most frequently methylated CpG sites.

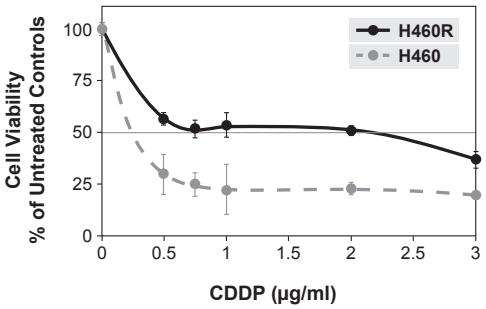
a



c



b

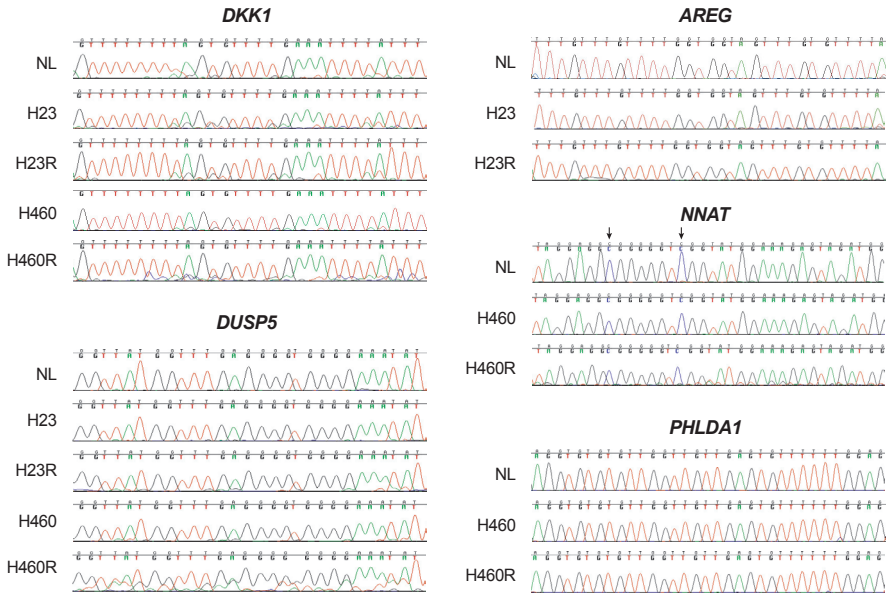


d

	IC_{50} (µg/ml) ± SD	CDDP RI	P-value
H23	0.52 ± 0.11	-	-
H23R	2.12 ± 0.33	4.07	<0.001
H460	0.29 ± 0,07	-	-
H460R	1.13 ± 0,49	3.89	0.002
41S	0.45 ± 0.12	-	-
41R	2.2 ± 0.2	4.8	<0.001

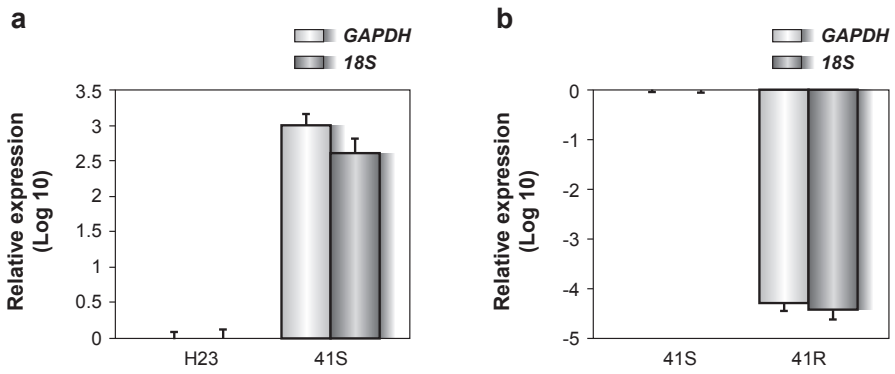
Supp. Figure 2

Supplementary Figure 2: Effect of cisplatin on cell viability. a, **b** and **c)** Viability curves showing the acquired resistance of H23, H460 and 41M cell lines; Cells were exposed for 72 h to each drug concentration. Data reported were normalized to the untreated control, which was set as 100%. Data represent the mean + SD of at least three independent experiments performed by quadruplicate at each drug concentration tested for every one cell line analyzed. **d)** IC_{50}, inhibitory concentration that kills 50% of cell population. RI; Resistance Index calculated as IC_{50} resistant / IC_{50} sensitive cell line. SD; Standard Deviation. P<0.01 was considered as a significant change in drug sensitivity (Student's t-test).



Supp. Figure 3

Supplementary Figure 3: Bisulfite sequencing of genes *DKK1, DUSP5, AREG, NNAT* and *PHLDA1. DKK1* and *DUSP5* were identified in both NSCLC cell lines while *AREG* and *PHLDA1* were identified specifically in the cell lines H23 and H460 respectively. Their methylation status was unmethylated as it can be observed in all samples tested with the presence of T instead of C previous G. However the gene *NNAT,* identified in the cell line H460, was methylated in normal lung DNA and in the cell lines H460 parental (H460) and resistant (H460R) as it is indicated by the presence of C instead of T in the positions pointed by the arrows.



Supp. Figure 4

Supplementary Figure 4: Relative expression levels of *IGFBP-3*, measured by quantitative real time PCR, in the cell lines H23, 41S and 41R, represented in Log 10 scale; *GADPH* (light grey) and *18S* (dark grey) were used as endogenous control genes. Each bar represents the combined relative expression of two independent experiments measured by triplicate. **a)** H23 is used as calibrator. The *IGFBP-3*-unmetylated 41S cell line is overexpressed by 1000 times compared with the *IGFBP-3*-semimethylated H23 cell line, confirming the tightly concordance between expression and gene methylation status. **b)** 41S is used as calibrator. The methylation of *IGFBP-3* in the 41R cell line went along with a decrease in gene expression of more than 10000 folds compared with the parental 41S.

PRIMERS		*IGFBP-3*	*ARRDC4*	*DKK1*	*DUSP5*	*AREG*
RT-PCR	SENSE	5-GTCAACGCTAGTGCCGTCAG-3	5-TATTCCAGCGGCGAGACAG-3	5-TCGGTTCTCAATTCCAACGCTA-3	5-TATCCTGAGTGTTGCGTGGATG-3	5-TCTCCACTCGCTCTTCCAAC-3
	ANTISENSE	5-GCTCTGAGAC TCGTAGTCAAC-3	5-GCTGTAATAAAATGATGCCTTC-3	5-TCCRAACACCAAACCCAAACTCT-3	5-GGAGCTAATGTCAGCCGTGTG	5-GCTGTAATAAAATGATGCCTTC-3
Bisulfite Sequencing	SENSE	5-AGAAGTAAGTTTGGAAAGGGGYGA-3	5-TATTTATTTTTTTTYGGAGTATTGTAGA-3	5-GTTGGTAGTTTTTATTTYGAAGGTGA-3	5-GYGYGGAAGAGAGAAYGAAGA-3	5-GTATATTTGGGTGTTAGCGTTTTAGA-3
	ANTISENSE	5-ACCCCRACACTCCAAACCACCT-3	5-TCCRAACACCAAACCCAAACTCT-3	5-CRCTACRCCCAAAACCATCATCT-3	5-CCRCCTCCTTACRAAACATCT-3	5-AAAAACCAAACTCAAAAAACAAAACCT-3
Bisulfite Sequencing	SENSE	GGTGCTGAGTTGGCCAGGAGTGA	5-GGTGGGAGYGGTTAATGAAATGA-3		5-ATATAGAAAAGTGGAGAAAATAGTA-3	
	ANTISENSE	GAATCCAGGCAGGAAGCGGCTGATCCT	5-TCCRAACACCAAACCCAAACTCT-3		5-AAAACRCCTCTTCRTTCTCTCT-3	
MSP Unmethylated	SENSE	5-AGAAAGTTTTATGAGGTATATATGA-3				
	ANTISENSE	5-CACTCTCAAAATAAAATCTCCCT -3				
MSP Methylated	SENSE	5-TTTTACGAGGTATATACGAATGC -3				
	ANTISENSE	5-TCTCGAAATAAAATCTCCCTACG-3				

PRIMERS		*GDF15*	*NNAT*	*PHLDA1*	*S100A2*	*GAPDH*
RT-PCR	SENSE	5-GACCCTCAGAGTTGCACTC-3	5-CTCTTTCTCGACCACCCACCTACC-3	5-AGGAAGGGCTGCTGCTTATC-3	5-AGCTTTGTGGGGGAGAAAGT-3	5-GAGAGACCCTCACTGCTG-3
	ANTISENSE	5-CTTGCAAGGCTGAGCTGAC-3	5-CACAGGAGCACCTGATGATAC-3	5-GTGCAGTTCCTTGAGCTTGAC-3	5-ATCCATGGCAGGAAGTCAAG-3	5-GATGGTACATGACAAGGTGC-3
Bisulfite Sequencing	SENSE	5-TAGTATTGGTTAGTTTTAGTTTAGA-3	5-GTAGGTTAGGGATTGGGGAGA-3	5-TATTTTTTGAGGTTGTTAGTAGA-3	5-CGTTTTGTYGTTTAGGTTGGA-3	
	ANTISENSE	5-ATCTCTAAAATTTCACTTACC TTCT-3	5-CGAAACCCCRTCAAACTTACCT-3	5-RCRACRCTTTTAAATAAACCATCT-3	5-AAAACCCCTAAACTAAAATATCC-3	

Supp. Table 1

Supplementary Table 1: Sequences of the RT-PCR, BS and MSP primers designed for the nine genes selected in this study together with the endogenous GAPDH gene.

Samples	Histo. Type	TNM	Stage	Age	Gender	CDDP	IC_{50} (µg/ml)	MSP	DFS	Relapse	GS	Exitus
1	SCCA	T1N0	1A	48	M	R	>100	U	15	Yes	15	Yes
2	Adeno	T1N0	1A	71	M	R	>100	M	30	No	30	No
3	Large cell	T2N1	2B	59	M	R	100	M	4	Yes	6	Yes
4	SCCA	T2N0	1B	59	M	R	>100	M	1	No	1	Yes
5	Adeno	T2N0M0	1B	67	M	R	>100	M	48	No	48	No
6	SCCA	T2N1	2B	58	M	R	>100	M	61	No	61	No
7	SCCA	T3N0M0	2B	65	M	R	>100	U	14	No	14	No
8	SCCA	T2N0M0	1B	79	M	R	>100	M	17	No	17	No
9	Adeno	T1N0M0	1A	79	M	R	>100	U	17	No	17	No
10	SCCA	T1N0M0	1A	65	M	R	>100	U	51	No	51	No
11	SCCA	T1N0	1A	63	M	R	>100	M	16	Yes	18	Yes
12	Large cell	T1N0M0	1A	78	M	R	>100	M	12	Yes	18	Yes
13	SCCA	T2N0	1B	77	M	R	>100	M	1	No	1	No
14	Large cell	T1N0M0	1A	71	M	R	>100	M	51	No	51	No
15	Adeno	T2N0M0	1B	69	M	R	90	M	17	No	17	No
16	Adeno	T1N0M0	1B	70	M	R	40	M	17	No	17	No
17	SCCA	T1N1M0	2A	75	M	R	30	M	18	No	18	No
18	SCCA	T2N0M0	1B	78	M	R	14	U	7	Yes	17	Yes
19	SCCA	T2N0	1B	65	M	R	10	M	35	Yes	35	Yes
20	Adeno	T2N0	1B	68	M	S	5	U	10	No	10	No
21	SCCA	T2N0M0	1B	58	M	S	2	U	17	No	17	No
22	Adeno	T2N0M0	1B	60	M	S	2	U	1	No	1	Yes
23	Adeno	T3N0	2B	73	M	S	2	U	20	Yes	22	Yes
24	SCCA	T2N1	2B	61	M	S	1	U	1	No	1	Yes
25	SCCA	T2N0	1B	61	M	S	0.5	U	62	No	62	No
26	SCCA	T3N0	2B	58	M	S	0.09	U	1	No	1	No
27	SCCA	T2N0	1B	69	M	S	0.01	U	72	No	72	No
28	SCCA	T2N0	1B	74	M	S	0.01	M	72	No	72	No
29	SCCA	Unknown	Unknown	75	M	S	0.01	U	1	No	1	No
30	SCCA	T3N0	2A	64	M	S	0.008	M	1	No	1	Yes
31	SCCA	T2N1	2B	54	M	S	0.008	U	1	No	1	No
32	SCCA	T2N1	2B	58	F	S	0.007	U	1	Yes	2	Yes
33	Adeno	T2N1	2B	49	F	S	0.006	U	12	Yes	64	No
34	Large cell	T2N0	1B	74	M	S	0.005	U	60	No	60	No
35	SCCA	T2N1	2B	60	M	S	0.005	U	60	No	60	No
36	Adeno	T2N1	2B	58	M	S	0.005	U	3	Yes	5	Yes

Supp. Table 2

Supplementary Table 2: Clinicopathological, stages, age, gender, CDDP resistance and hypermethylation of *IGFBP-3* detection data of 36 NSCLC patients. NOTE. Age, years; Histo type SCCA, squamous cell carcinoma; Grade: American Joint Committee on Cancer; Stage: American Joint Committee on Cancer stage grouping; CDDP; R, resistant to CDDP, S sensitive to CDDP; IC_{50}, drug concentration that kills 50% of cell population after 72h of CDDP expousure. MSP; M methylated; U, unmethylated for *IGFBP-3;* DFS, Desease free survival; GS, Global survival.

Capítulo 2

IGFBP-3 methylation-derived deficiency mediates the resistance to cisplatin through the activation of the IGFIR/Akt pathway in non-small cell lung cancer

Una vez observado que la hipermetilación del promotor de *IGFBP-3* estaba implicada en el desarrollo de resistencia a CDDP en CNMP, quisimos estudiar qué vías de señalización estaban involucradas en este proceso. La proteína IGFBP-3 se une al factor de crecimiento IGF-I impidiendo la unión de éste a sus receptores, y previsiblemente inhibiendo la activación de AKT y la supervivencia celular; por ello decidimos estudiar el estado de activación de la vía de señalización de PI3K/AKT y de los receptores EGFR e IGF-IR en líneas celulares tumorales.

En primer, se utilizó para el estudio el grupo de las tres líneas celulares tumorales sensibles/resistentes a CDDP, H460S/R, H23S/R y 41S/R, que tenían un grado diferente de metilación del promotor de *IGFBP-3,* y se estudió en ellas la activación de AKT. Así se observó que las líneas celulares 41R y H23R, que tenían el promotor de *IGFBP-3* más densamente metilado y por tanto bajos niveles de expresión de dicho gen, presentaban la proteína AKT fosforilada en condiciones basales y/o a lo largo del tratamiento con CDDP, fenómeno que no ocurría en las líneas celulares 41S y H23S, cuyo promotor de *IGFBP-3* no estaba metilado o bien presentaba niveles muy bajos de metilación. En la línea celular control negativo H460R, en la que la resistencia no parece mediada por la metilación del gen *IGFBP-3,* no se encontraron cambios en la activación de AKT. Para dilucidar si la respuesta a CDDP mediada por la expresión de *IGFBP-3* se producía a través de cambios en la actividad de AKT, se sobreexpresó el gen de *IGFBP-3* en la línea celular 41R y se estudió por una parte, la viabilidad celular a CDDP y por otra la activación de AKT. Se observó, que el aumento de la expresión de *IGFBP-3* incrementaba la sensibilidad a CDDP mostrando un grado intermedio de sensibilidad entre el de las líneas celulares 41S y 41R. Además los niveles de activación de AKT disminuían en la línea 41R con *IGFBP-3* sobreexpresada. Para verificar el papel de AKT en la resistencia adquirida a CDDP, se inhibió la expresión de AKT mediante el uso de un ARN de interferencia y se inhibió también su activación con el inhibidor de PI3K, LY294002. Así se observó que cuando se inhibía la expresión de AKT en las células 41R se producía un incremento en la sensibilidad a CDDP, que en algunas dosis del fármaco se igualaba a la línea 41S y que la inhibición de la fosforilación de AKT en las células 41R provocaba un cambio en la mortalidad celular en respuesta a CDDP si se comparaban con las células 41R sin tratar con el inhibidor.

Posteriormente se estudió la participación de ambos receptores, EGF e IGF-I en la activación de AKT, y se observó que el receptor IGF-IR se encontraba activado en la línea celular 41R en condiciones basales y que esta activación se mantenía en todas las dosis de CDDP ensayadas, efecto que no se observó en la línea celular 41S, donde el IGF-IR sólo se encontraba fosforilado cuando las células eran tratadas con las dosis más altas de CDDP. El receptor de EGF por el contrario no parecía activarse en ninguna de las dos líneas celulares y además sus niveles de expresión eran muy bajos en la línea celular 41R. Para comprobar que la activación de IGF-IR observada en la línea celular 41R era consecuencia de los niveles disminuidos de *IGFBP-3,* se procedió a la sobreexpresión de su ADNc en dichas células, observándose que la sobreexpresión de *IGFBP-3* disminuía la fosforilación de IGF-IR comparado con los niveles de fosforilación del receptor en las células 41R. Sin embargo no se observó ningún cambio en el receptor EGFR. Para determinar que los resultados obtenidos hasta ahora no eran específicos de las líneas celulares 41S/R, se utilizaron las células tumorales PANC-1, H1299, H727 y HT29 que presentaban el promotor de *IGFBP-3* metilado y diferentes IC_{50} al agente CDDP; la sobreexpresión de *IGFBP-3*

en las líneas celulares H1299 y PANC-1 dio lugar a un perfil de activación de AKT e IGF-IR parecido al observado en la línea celular 41R. Hasta aquí, nuestros datos sugieren que la pérdida de expresión de *IGFBP-3* por la metilación de su promotor en células tumorales tratadas con CDDP puede activar la vía de PI3K/AKT a través de la vía de señalización del factor de crecimiento IGF-I/IGF-IR, induciendo resistencia a CDDP.

Finalmente, a partir de las muestras de pacientes de CNMP que se habían utilizado en el manuscrito anterior, estudiamos si los datos obtenidos sobre sus IC_{50} y el estado de metilación del promotor de *IGFBP-3* correlacionaban también con la activación/fosforilación de AKT, IGF-IR y EGFR. Observamos en primer lugar la existencia de una activación predominante de IGF-IR (32%) pero no de EGFR (16%); además un 75% de los pacientes que tenían fosforilado IGF-IR presentaban también activación de AKT, y un 87% de las muestras que tenía el receptor de IGF-I activo eran resistentes a CDDP. Así se desarrolló un panel predictivo, que considera que una muestra necesita al menos dos modificaciones de las cuatro siguientes, metilación del promotor de *IGFBP-3,* activación de IGF-IR, activación de EGFR y activación de AKT, para conseguir un cambio de fenotipo en términos de respuesta a CDDP. Con estos datos se realizó un test de diagnóstico basado en la probabilidad de aparición de resistencia a CDDP, que correlacionaba los diferentes parámetros con una exactitud y una especificidad de 0,84 y 0,9 respectivamente.

Oncogene (2012), 1–10

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ORIGINAL ARTICLE

IGFBP-3 methylation-derived deficiency mediates the resistance to cisplatin through the activation of the IGFIR/Akt pathway in non-small cell lung cancer

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Although many cancers initially respond to cisplatin (CDDP)-based chemotherapy, resistance frequently develops. Insulin-like growth factor-binding protein-3 (*IGFBP-3*) silencing by promoter methylation is involved in the CDDP-acquired resistance process in non-small cell lung cancer (NSCLC) patients. Our purpose is to design a translational-based profile to predict resistance in NSCLC by studying the role of *IGFBP-3* in the phosphatidyl inositol 3-kinase (PI3K) signaling pathway. We have first examined the relationship between *IGFBP-3* expression regulated by promoter methylation and activation of the epidermal growth factor receptor (EGFR), insulin-like growth factor-I receptor (IGFIR) and PI3K/AKT pathways in 10 human cancer cell lines and 25 NSCLC patients with known *IGFBP-3* methylation status and response to CDDP. Then, to provide a helpful tool that enables clinicians to identify patients with a potential response to CDDP, we have calculated the association between our diagnostic test and the true outcome of analyzed samples in terms of cisplatin IC_{50}; the inhibitory concentration that kills 50% of the cell population. Our results suggest that loss of *IGFBP-3* expression by promoter methylation in tumor cells treated with CDDP may activate the PI3K/AKT pathway through the specific derepression of IGFIR signaling, inducing resistance to CDDP. This study also provides a predictive test for clinical practice with an accuracy and precision of 0.84 and 0.9, respectively, ($P = 0.0062$). We present a biomarker test that could provide clinicians with a robust tool with which to decide on the use of CDDP, improving patient clinical outcomes.

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Keywords: NSCLC; cisplatin resistance; IGFIR; IGFBP-3; hypermethylation

INTRODUCTION

Advanced non-small cell lung cancer (NSCLC), accounts for one out of six cancer-related deaths worldwide,[1] mainly secondary to late diagnosis and intrinsic or acquired resistance to available therapies. Platinum-induced DNA hypermethylation may be one of the mechanisms involved in the development of resistant phenotypes by inactivating genes required for drug cytotoxicity.[2,3] Unfortunately, in disseminated patients without epidermal growth factor receptor (EGFR) mutations or ALK-EML4 expression in their tumors, the standard treatment is based on platinum doublets but clinical probability to achieve an objective response or increased survival after a cisplatin (CDDP)-based therapy for metastatic disease is $<40\%$.[4]

Insulin-like growth factor-binding protein-3 (*IGFBP-3*) is a member of the insulin-like growth factor-binding protein family that binds IGF-I, blocking its mitogenic and antiapoptotic actions. In the absence of IGFBP-3, IGF-I binds and activates insulin-like growth factor-I receptor (IGFIR) and EGFR, commonly overexpressed in NSCLC,[5,6] which signal through the PI3 kinase (PI3K)/Akt pathway, having a crucial role in cell growth, proliferation and survival.[7] In addition, genetic and epigenetic alterations account in multiple genes in the pathway.[8,9] Furthermore, aberrant hypermethylation is a common epigenetic mechanism for the silencing of *IGFBP-3* in several tumor types, including NSCLC.[10–12]

The IGFBP-3/IGF-I/IGFIR axis exemplifies redundant routes to induce resistance to many drugs.[13] First, many proteins belonging to this pathway mediate chemotherapy resistance in NSCLC through the activation of Akt/mTOR (mammalian target of rapamycin) signaling.[14,15] Second, a model of acquired resistance to EGFR tyrosine kinase inhibitors showed *IGFBP-3* downregulated without changes in IGF-I and II levels, suggesting that EGFR tyrosine kinase inhibitor-resistant cells adopted IGFIR pathway in addition to EGFR/ErbB-3 to activate PI3K,[5] although possible mechanisms underlying *IGFBP-3* downregulation and further IGFIR activation were not described. Third, we have reported that reduction of *IGFBP-3* expression by promoter methylation is involved in CDDP resistance process in NSCLC;[16] probably as a result of a higher amount of IGF-I available to bind its canonical receptor. All these data suggest that an isolated alteration is not enough to induce resistance to cancer therapies.

In order to design a translational-based profile to predict resistance in NSCLC, more comprehensive information is necessary regarding the IGFBP-3/IGFI/IGFIR axis. To evaluate this hypothesis, we have used cellular models of CDDP-acquired resistance with different *IGFBP-3* promoter-methylation status to study the alterations in the PI3K signaling pathway through EGFR and IGFIR. Then we have tested these data into a cohort of 25 NSCLC specimens with previously known *in vitro* CDDP sensitivity

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to calculate minimum requirements to predict resistance. Finally, we have computed likelyhood ratios and nomograms for clinical practice. In this regard, our results suggest that our approach that combines *IGFBP-3* methylation status together with the AKT, IGFIR and EGFR activation enables the identification of CDDP responders and suggest future drugs combinations to optimize CDDP-based NSCLC therapy.

RESULTS

IGFBP-3 methylation induces the phosphorylation of AKT in CDDP-resistant human cancer cell lines

To determine whether the resistance to CDDP mediated by IGFBP-3 promoter-methylation[16] correlates with the activation of the PI3K/AKT cellular pathway, we used the matched CDDP-sensitive/resistant cell lines 41S/41R, H460S/H460R and H23S/H23R.[16] We first analyzed the IGFBP-3 expression that was dramatically decreased in the resistant cell line 41R, whereas no changes were observed in H460R cells, compared with their parental lines. Additionally, H23S cells (that harbors a semimethylated IGFBP-3 promoter[16]) showed less IGFBP-3 expression than 41S and H460S cells, confirmed by the decreased ratio between IGFBP-3/GAPDH signals. The matched H23R cells harbor a more densely methylated promoter,[16] in line with the decrease observed in the gene expression (Figure 1a, left panel). We then confirmed the presence of IGFBP-3 promoter hypermethylation in 41R and H23R cells by methylation-specific PCR, whereas it was unmethylated in H460R cells, (Figure 1a, right panel). Supporting our hypothesis

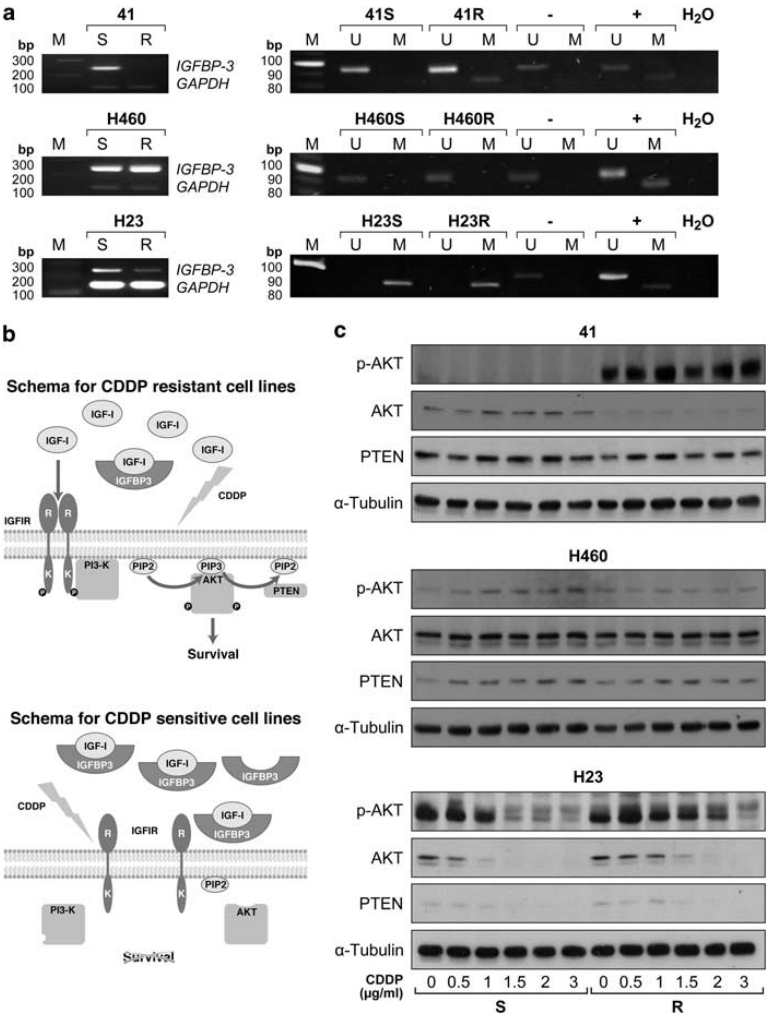


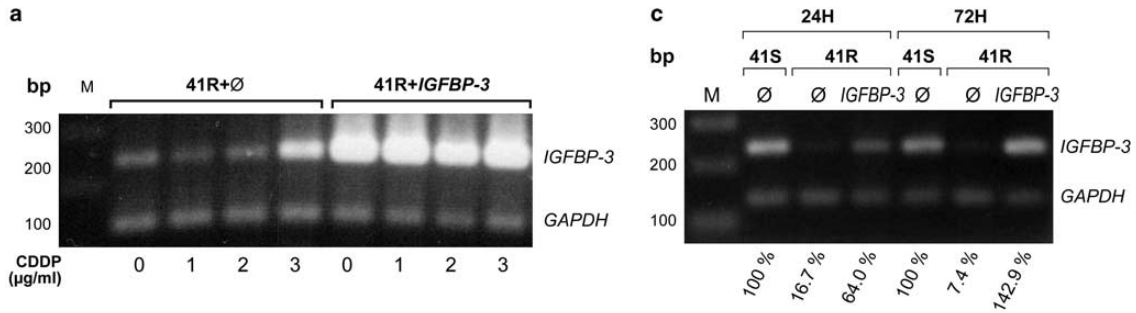
Figure 1. Association between *IGFBP-3* promoter methylation status, gene expression levels and AKT activation. (**a**) Paired-matched cell lines 41S/R, H460S/R and H23S/R present different *IGFBP-3* expression profile measured by RT–PCR (left panel) and also different gene methylation status (right panel). GAPDH mRNA was co-amplified as a loading control and the *IGFBP-3* promoter methylation was analyzed using methylation-specific PCR (MSP). The presence of a PCR product in the methylated lane (M) indicates methylated alleles for *IGFBP-3*. Tumor cell lines H1299 and A549 were used as positive and negative controls (NC), respectively. (**b**) Schemas representing the different status of the IGFIR/PI3K/AKT pathway proposed for the CDDP-resistant or -sensitive cell lines, regarding the *IGFBP-3* level. (**c**) Cells were seeded and 24 h later incubated in serum-depleted medium for 24 h, then treated with the indicated CDDP doses for 6 h. Total cell protein (20 μg) was subjected to WB and the membranes were hybridized with antibodies against p-AKT, AKT, PTEN and α-tubulin as a loading control.

(Figure 1b), there was a strong phosphorylation of AKT in all CDDP doses tested in the resistant cell line 41R (Figure 1c) that was not due to increased AKT basal level, and cell line H23R presented a wider CDDP dose-response activation of AKT (2 μg/ml) in comparison with the H23S cells (1 μg/ml) (Figure 1c). In addition, no changes in either p-AKT or AKT levels were visible in the cell line H460R, compared with the parental cells. The phosphatase and tensin homolog (PTEN) levels were not affected in 41S/R and H460S/R cells and were undetectable in H23S/R cells.

The response to CDDP is mediated by IGFBP-3 expression through the AKT function

To confirm that IGFBP-3 expression was linked to CDDP sensitivity through AKT-phosphorylation, we chose 41R cells to again induce the IGFBP-3 expression through both gene overexpression and epigenetic reactivation.

IGFBP-3 was successfully reexpressed in 41R cells among different CDDP doses tested (0, 1, 2 and 3 μg/ml) (Figure 2a), lowering the AKT phosphorylation to intermediate levels between 41R and 41S cells transfected with the empty vector (Figure 2b). PTEN levels were not modified, as previously observed (Figure 1d). We then examined whether the decrease in AKT activation induced changes in sensitivity to CDDP; IGFBP-3 was reexpressed in 41R cells 24 h after transfection, an effect remaining 72 h later, with 143% efficiency, compared with 41R cells transfected with the empty vector (Figure 2c). IGFBP-3 overexpression induced an increase in CDDP sensitivity compared with the control-transfected cells, resulting in a decrease in the IC50 value in response to CDDP (1.15 μg/ml vs 1.91 μg/ml; $P<0.001$) (Figure 2d). Those cells (41R + IGFBP-3) showed an intermediate degree of sensitivity between 41S and 41R cells transfected with the negative control (NC), with a resistance index lower than 41R + pCMV5 cells (1.4 vs 2.4; $P<0.001$) in which *IGFBP-3* is completely



	IC50 (μg/ml) ± SD	CDDP RI	P-value
41S+Ø	0.81 ± 0.07	_	_
41R+*IGFBP-3*	1.15 ± 0.19	1.4	<0.005 (41S Ø)
			<0.001 (41R Ø)
41R+Ø	1.91 ± 0.12	2.4	<0.001 (41S Ø)

Figure 2. Effect of *IGFBP-3* overexpression on AKT phosphorylation and cell sensitivity to CDDP. (**a**) *IGFBP-3* expression by RT–PCR after transfection with 1.5 μg of human *IGFBP-3* cDNA or empty vector (Ø) in 41R cells treated with four different doses of CDDP. GAPDH mRNA was co-amplified as a loading control. (**b**) 41R and 41S cells were transiently transfected with pCMV5 (Ø) or with pCMV5-*IGFBP-3* vectors, then incubated in serum-depleted medium for 24 h, followed by CDDP treatment at indicated doses for 6 h. Total cell protein (20 μg) was subjected to WB and the membranes were hybridized with antibodies against p-AKT, AKT, PTEN and α-tubulin as a loading control. (**c**) *IGFBP-3* mRNA levels measured by RT–PCR in 41R and 41S cells transfected with 1.5 μg of human *IGFBP-3* cDNA or empty vector (Ø) at 24 and 72 h after transfection. cDNA quantity was estimated by PCR amplification of the *GAPDH* gene using ImageJ 1.37V N (http://www rsb.info.nih.gov/ij/) (**d**) Cell survival of 41R cells transfected with empty vector (41R + pCMV5 (Ø)) or human *IGFBP-3* cDNA (41R + *IGFBP-3*) and 41S cells transfected with the empty vector (41S + pCMV5 (Ø)), to CDDP at six different test drug concentrations. Data were normalized to each untreated control, set to 100%. Data represent the mean ± s.d. of at least three independent experiments performed with 8 wells at each drug concentration for every cell line analyzed. Data represent the *IC50*, 48 h after CDDP exposure; the CDDP RI, or resistance index to CDDP was calculated as (*IC50* from 41R + *IGFBP-3* or 41R + Ø)/(*IC50* 41S + Ø) ± the s.d. or s.d. $P<0.005$ was considered a significant change in drug sensitivity (Student's *t*-test). M, marker; bp, base pair.

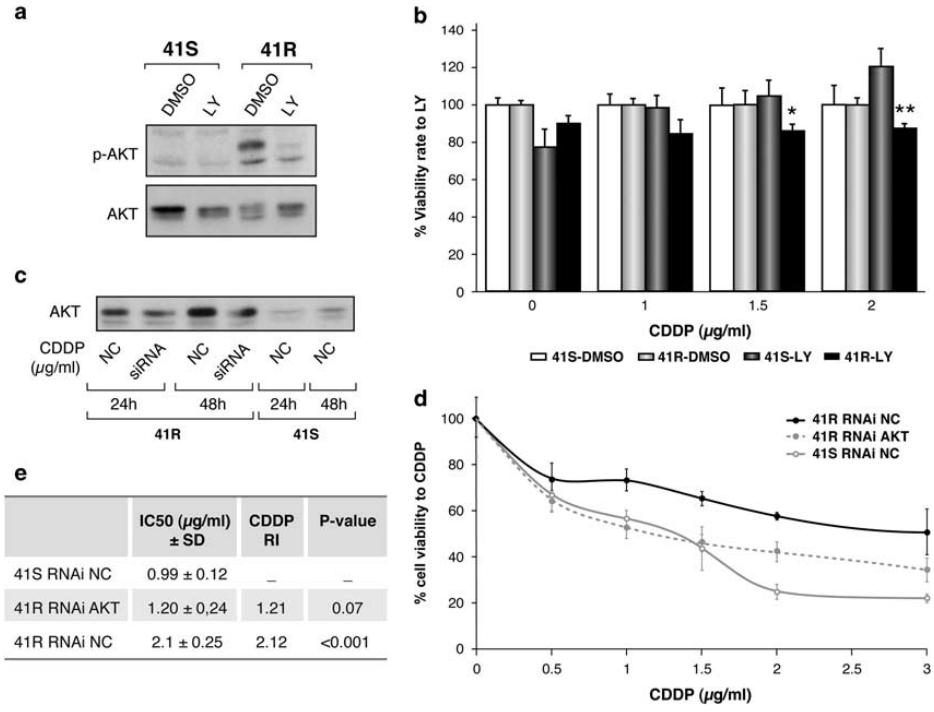
silenced by promoter methylation. The epigenetic reactivation treatment was also effective in reexpressing IGFBP-3 in 41R cells from the first dose of CDDP exposure (Supplementary Figure 1, upper panel), increasing the sensitivity to CDDP and reaching intermediate levels between 41S and 41R cell lines (Supplementary Figure 1, lower panel).

Next, to verify the role of AKT in the acquired resistance to CDDP, we first inhibited the AKT activation using LY294002, and then the AKT expression by small interfering RNA (siRNA), in the cell lines 41S/41R. As expected, 41S cells were not affected by LY294002 treatment, as this cell line lacks basal phosphorylation of AKT; however, in 41R cells treatment reduced the p-AKT levels (Figure 3a), inducing a significant change in cell mortality at 1.5 and 2 μg/ml of CDDP ($P<0.05$ and $P<0.001$, Figure 3b). In addition, AKT expression was inhibited in 41R cells 24 h after siRNA transfection, an effect remained after 48 h, compared with cells transfected with control-RNA interference (RNAi) (NC) (Figure 3c). AKT silencing in resistant cells (41R RNAi AKT) induced an increase in CDDP sensitivity, compared with cells transfected with NC (41R RNAi NC) (Figure 3d), resulting in a significant decrease in the IC50 value in response to CDDP (1.20 μg/ml vs 2.10 μg/ml, $P<0.001$). Regarding CDDP sensitivity, these cells showed no significant differences from the 41S cells transfected with NC (41S RNAi NC) ($P=0.07$) (Figure 3e).

IGFBP-3 expression regulates the IGFIR/AKT pathway inducing cell sensitivity to CDDP

To assess which receptor is involved in the observed AKT phosphorylation, we measured p-EGFR, EGFR, p-IGFIR and IGFIR protein levels in response to the increasing doses of CDDP in our panel of three matched sensitive/resistant cell lines, together with EGF or IGFI treatment as positive controls.

EGF stimuli induced the activation of EGFR only in 41S cells. In addition, in 41R cells we found lower levels of EGF receptor in all CDDP doses tested (Figure 4a). The IGFI stimuli induced the activation of the IGFIR only in the resistant cell line 41R. Furthermore, IGFIR was active at basal conditions in 41R cells (0 μg/ml CDDP), activation maintained at all CDDP doses tested, whereas it was activated in 41S only at high doses (2–3 μg/ml) (Figure 4a). Activation of the IGFIR observed in 41R cells shrinks when IGFBP-3 is overexpressed (Figure 4b); effect, observed in all CDDP doses tested. Additionally, no changes were observed in either the EGFR levels or in its activation when IGFBP-3 is overexpressed (Figure 4b). As expected, no changes in EGFR or IGFIR activation were observed in H460 and H23 cells, as no noticeable changes in IGFBP-3 expression or in AKT activation were initially found when cells developed an acquired resistance to CDDP (Supplementary Figure 2).



	IC50 (μg/ml) ± SD	CDDP RI	P-value
41S RNAi NC	0.99 ± 0.12	_	_
41R RNAi AKT	1.20 ± 0,24	1.21	0.07
41R RNAi NC	2.1 ± 0.25	2.12	<0.001

Figure 3. Effect of AKT inhibition by LY294002 inhibitor and AKT siRNA on cell sensitivity to CDDP. (**a**) 41S and 41R cell lines were treated either with the LY 294002 inhibitor or with dimethyl sulfoxide (DMSO) as negative control for 1.5 h in order to test by WB the inhibition of AKT phosphorylation in 41R cells. (**b**) Simultaneously, same experimental groups were treated with DMSO or LY 294002 as described in **a**, followed by CDDP treatment at 0, 1, 1.5 and 2 μg/ml to measure the cell viability rate to LY. *$P<0.05$, **$P<0.001$ (Student's *t*-test). (**c**) *AKT* protein levels in 41R and 41S cells transfected with siRNA against *AKT* or with siRNA-NC at 24 and 48 h after transfection, as internal control for the study described in **d**. Total cell protein (20 μg) was subjected to WB and then membranes were hybridized with antibodies against AKT and α-tubulin as a loading control. (**d**) Viability curves of 41R cells transfected with siRNA against *AKT* (41R RNAi *AKT*) or with NC (41R RNAi NC) and 41S cells transfected with NC (41S RNAi NC) to CDDP at six different test drug concentrations. Data were normalized to each untreated control, set to 100%. Data represent the mean ± s.d. of at least three independent experiments performed with 8 wells at each drug concentration for every cell line analyzed. (**e**) Data represent the *IC50*, , 48 h after CDDP exposure; the CDDP or resistance index (RI) to CDDP was calculated as (*IC50* from 41R RNAi *AKT* or 41R RNAi NC)/(*IC50* 41S RNAi *AKT*) ± the s.d. or s.d. $P<0.001$ was considered a significant change in drug sensitivity (Student's *t*-test).

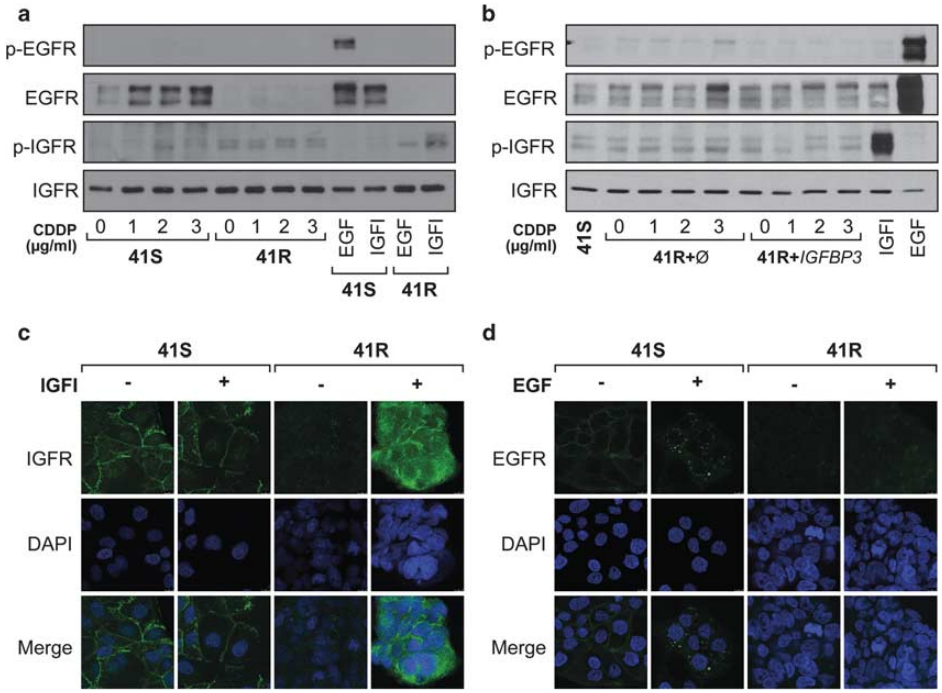


Figure 4. Differential activation of EGF and IGFI receptors in 41S and 41R cell lines. (**a**) The protein levels of pEGFR, EGFR, pIGFIR and IGFIR were measured by WB. Cells were seeded and 24 h later incubated in serum-depleted medium for 24 h, then treated with the indicated CDDP doses for 6 h or with EGF or IGFI for 30 min as control stimuli. Total cell protein (20 μg) was subjected to WB and the membranes were hybridized with antibodies against pEGFR, pIGFIR, EGFR and IGFIR. (**b**) The 41R cell line were transiently transfected with pCMV5 (Ø) or with pCMV5-*IGFBP-3* vectors, then cells were treated as in **a**. (**c** and **d**) IGFIR and EGFR cellular localization analyzed by immunofluorescence in 41S and 41R cell lines. Cells were grown on coverslips, 24 h later cells were shifted into medium containing 0.5% fetal bovine serum for 16 h, then cells were treated or not with IGFI or EGF as positive controls for 30 min, subsequently the coverslips were fixed and incubated with antibodies against IGFIR or EGFR, and then with a secondary antibody conjugate (fluorescent dye alexa fluor 488, Invitrogen, Carlsbad, CA, USA). The immunofluorescence was visualized with a confocal microscope.

Using immunofluorescence we also found that serum-starved 41S cells showed prominent membrane IGFIR and partial receptor internalization after IGFI stimuli. However, its distribution was affected in 41R cells, in which we detected mainly the intracellular IGFIR that strongly increased with IGFI treatment (Figure 4c). IGFI also induced IGFIR nuclear translocation in 41R cells, according to previous data.[17] We next confirmed some basal EGFR membrane presence only in 41S cells that translocate into the cytoplasm when EGF was added (Figure 4d). In addition, we did not find any changes in the EGFR mRNA levels between 41S and 41R cells (data not shown).

To determine that these results were not specific for the cell lines 41S/41R, we used the cell lines PANC-1, H1299, H727 and HT29 that harbor a methylated promoter for the IGFBP-3 gene and different IC50 to CDDP.[16] We first confirmed that all cell lines lacked or expressed IGFBP-3 at a low level (Ø) (Supplementary Figure 3a); next, the IGFBP-3 reexpression induced a severe decrease in cell survival compared with cell lines transfected with the empty vector (Supplementary Figure 3b).

We also found that IGFBP-3 overexpression in H1299 lung cancer cells (Figure 5a) was concomitant with a decrease in the IGFIR and AKT phosphorylation levels after CDDP treatment (Figures 5b and c). The same pattern was found in the cell line PANC1 for pIGFIR and pAKT levels at 2 and 3 μg/ml CDDP. At the same CDDP doses, pAKT levels in H727 cells also decreased after IGFBP-3 overexpression; however, IGFIR levels were almost undetectable, preventing the confirmation of any correlation. No changes in the activation of AKT and IGFIR were observed in HT29. In addition, the activation of EGFR was not modified in every cell line (Figures 5b and c).

Combination of IGFBP-3 promoter methylation with IGFIR, EGFR and AKT activation status predicts resistance or sensitivity to CDDP in NSCLC samples

As a translational approach, we further analyzed the correlation between IGFBP-3 methylation and the phosphorylation of IGFIR, EGFR and AKT in NSCLC primary tumors. In a previous study, we exposed 36 NSCLC fresh tumors to different CDDP concentrations and analyzed their IGFBP-3-methylation status, comparing the results with the clinical history.[16] According to Receiver operating characteristic (ROC) methodology, the IC50 index divided patients into two groups, regarding sensitivity or resistance to CDDP. From that cohort of NSCLC patients, we have assessed EGFR, IGFIR and AKT protein activation by immunohistochemical analysis in 25 surgical specimens (Supplementary Table 1 and Figure 6a). First, we found a predominant activation of IGFIR, but not EGFR, in NSCLC patients (32 vs 16%). In addition, 75% of patients with phosphorylated IGFIR, showed concomitant activation of AKT (Supplementary Table 1), and 87.5% of NSCLC patients with IGFI receptor activation (seven out of eight patients) were resistant to CDDP. Furthermore, the combination of IGFBP-3 methylation

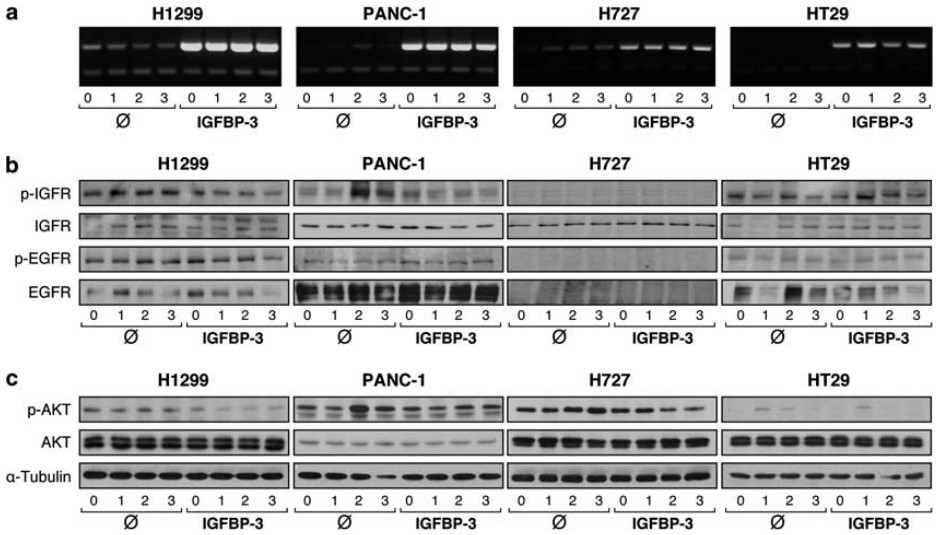


Figure 5. Correlation between *IGFBP-3* expression and IGFIR/AKT activation in different human cancer cell lines. The cell lines H1299, PANC-1, H727 and HT29 were transiently transfected with 1.5 μg pCMV5 (∅) or with pCMV5-*IGFBP-3* (*IGFBP-3*) vectors and 24 h later treated with CDDP at indicated doses for 6 h. Then RNA and protein extracts were obtained in parallel experiments. (**a**) *IGFBP-3* overexpression was confirmed by RT–PCR in all cell lines and GAPDH mRNA was co-amplified as a loading control. (**b** and **c**) Total cell protein (20 μg) was subjected to WB and then membranes were hybridized with antibodies against pIGFIR, IGFIR, pEGFR, EGFR, p-AKT, AKT and α-tubulin as a loading control.

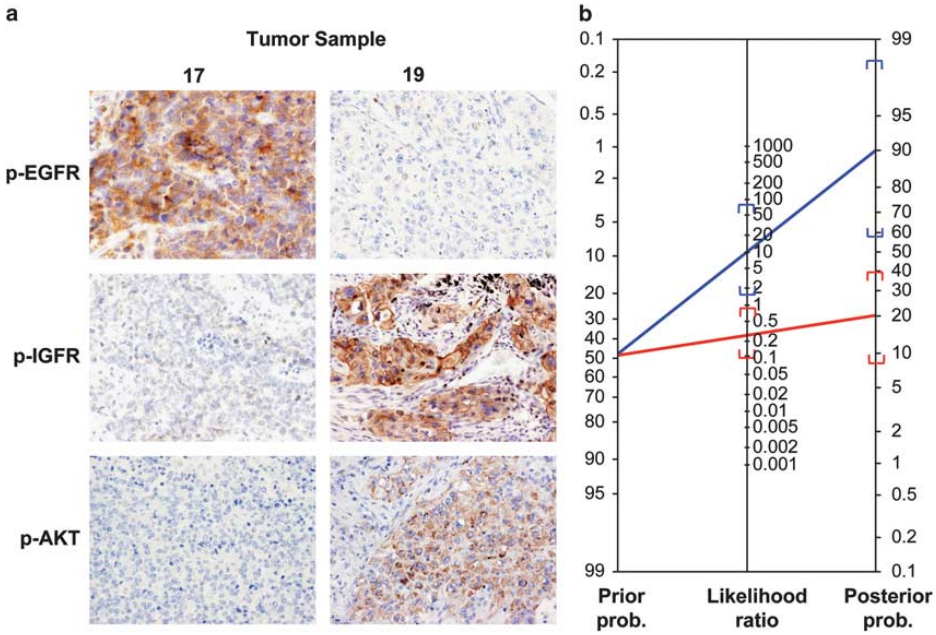


Figure 6. Combined use of EGFR, IGFIR and AKT protein phosphorylation together with *IGFBP-3* methylation status predicts a potential response to CDDP for NSCLC. A total of 25 NSCLC tissue samples were analyzed for stain localization and intensity. Immunohistochemistry (IHCs) were performed using polyclonal antibody that specifically recognizes p-EGFR or pIGFIR or p-AKT. Same samples were previously used for the analysis of *IGFBP-3* gene methylation status measured by MSP.16. (**a**) Example of IHC analysis in the examined tumor samples. Primary tumor 17 presents positive p-EGFR staining, without p-IGFIR and p-AKT staining, whereas patient number 19 presents positive p-IGFIR and p-AKT staining without significant p-EGFR stain. (**b**) Nomogram representing prior probability of positive-negative test (both of them with a 50% of chance for being positive or negative) and the post-test probability.

together with IGFIR and/or AKT phosphorylation only concurred in NSCLC patients resistant to CDDP, whereas no concomitant results from these parameters were found in CDDP-sensitive NSCLC patients.

With all these data we have developed a predictive panel, which considers that a sample needs at least two modifications to get a true phenotypic change in terms of CDDP response. Our predictive test found 15 patients with an apparent response to CDDP (S) as their harbored one or less events in our predictive panel. In all, 12 of them were truly sensitive to CDDP when confronted to IC_{50} data. On the opposite, 10 patients should have been resistant to CDDP accordingly to our test (R) and in fact only one failed this prediction (Supplementary Table 1). When all data were computed in a 2×2 contingency table, the accuracy and precision of this test was 0.84 and 0.9, respectively ($P = 0.0062$). When we consider that a sample needs at least two modifications to get a true phenotypic change in terms of CDDP response (two-sided $P = 0.0025$) with a positive post-test probability of 90% (positive likelihood ratio 9.75) and a negative post-test probability of 20% (Figure 6b).

DISCUSSION

The literature provides evidence that there is an increase of DNA hypermethylation after CDDP exposure *in vivo* and *in vitro*;[2,3,16,18–20] however, the molecular mechanisms still remain unclear. Even though it was thought that CDDP could not have epigenetic effects because it is a DNA crosslinking agent; however, significant evidence is emerging that links the loss of gene expression in NSCLC by DNA CpG methylation with CDDP resistance. In fact, pulsed exposure to CDDP has been shown to result in drug-induced DNA hypermethylation in lung adenocarcinoma (HTB-54), rhabdomyosarcoma (CCl-136) and ovarian human cancer cells (A2780),[18–20] an event that has also been reported *in vivo*.[2,3] It is well known that the metal toxicity induced by CDDP is because of reactive oxygen species production and the subsequent induction of oxidative stress.[21–23] Oxidative stress induces a complex cellular response, in which genetic and epigenetic alterations are involved. As an example, local generation of NO and superoxide anion O_2^- may activate RAS, which ultimately may induce the expression of DNA methyltransferases 1 and DNA methyltransferases 3B, and consequently the increase of DNA methylation.[24]

This DNA methylation may affect the expression of specific genes directly or as a result of microRNA silencing, in which mRNA targets are epigenetic regulators. This occurs with the miR29 family, which has been shown to be methylated in lung cancer and to directly target DNMT3A and 3B and indirectly DNA methyltransferases 1 expression.[25] In fact, the DNMTs family has been reported to be upregulated in CDDP-resistant clones established from the human cervix cancer cell line ME180.[26] This increase in expression seems to mediate the resistance to CDDP in mouse neuroblastoma, indicating that DNA methylation has a role in the occurrence of that resistance.[27]

In our case, loss of IGFBP-3 expression, through promoter hypermethylation, results in a reduction in tumor cell sensitivity to CDDP in NSCLC.[16] We cannot also discard possible aberrant expression of epigenetic regulators as histone demethylases or deacetilases. Histone deacetylases (HDACs) are important regulators of many oxidative stress pathways including those involved with both sensing and coordinating the cellular response to oxidative stress. In particular, aberrant regulation of these pathways by HDACs may have a critical role in cancer progression.[28] The HDAC4 and HDAC6 proteins are associated with specific regions of the IGFBP-3 gene, which coincides with histone H4 deacetylation on these regions that can ultimately silence gene expression.[29] In fact, we have shown that the use of histone deacetylation inhibitors in combination with demethylating agents restored IGFBP-3 expression.[16] This aberrant HDAC activity may be caused by the overexpression of metastasis-associated protein 1 and 2, which have been reported in NSCLC and have been shown to functionally associate with HDACs.[30] The hypoxic stress also induces an increase in diMe-histone H3 lysine 9 (H3K9), which is a critical epigenetic marker for gene repression and silencing and has an essential role in carcinogenesis. This increase may be mediated first by the inhibition of H3K9 demethylation processes,[31] probably secondary to the increase in the enzyme JMJD1B that demethylates trimethylated H3K9 to dimethylated H3K9. This process has been observed in renal cancer cells under hypoxic conditions.[32] Alternatively, the increase may be secondary to increased levels of DNA methyltransferases 1 after CDDP treatment, as it has been reported that dual DNA methyltransferases 1/3b knockout reduced the level of diMe-H3K9.[33]

However, for the majority of those aberrant epigenetics events, it is not particularly clear whether they are associated with responses to chemotherapy or they are occurring by chance owing to a methylator phenotype, or simply as random methylation events during platinum selection or DNA damage induction.[34] In an analogy to the concept of driver and passenger, methylation changes occurring during carcinogenesis could either represent drivers of chemoresistance based on their potential to provide the cell with a selective advantage, or passenger events, with no substantial impact on chemosensitivity.[35] Therefore, it is important to characterize each methylation event identified as a result of CDDP exposure, as many of them could not be related with chemotherapy resistance.

In the current study, we have used three paired sensitive/resistant cell lines that present a different *IGFBP-3* gene expression and promoter methylation profile to determine whether this resistance to CDDP is mediated by the activation of the PI3K/AKT cellular pathway. In fact, the resistance to CDDP in 41R and H23R cells was concomitant with the presence of IGFBP-3 hypermethylation and the decrease in IGFBP-3 expression. However, H460R cells harbored an unmethylated IGFBP-3 promoter, indicating that resistance to CDDP in these cells is independent of an IGFBP-3 epigenetic regulation, making them a perfect negative control for this study. Supporting our hypothesis, *IGFBP-3* hypermethylation correlated with a strong phosphorylation of AKT in 41R cells. Interestingly, H23S that harbors a semimethylated promoter showed basal AKT phosphorylation, that resulted in a wider CDDP dose-response activation of the protein when the cell line acquires a more densely hypermethylated promoter[16] as a result of resistance establishment (H23R). As expected, no changes were visible in the cell line H460R. We discarded a role for PTEN mediating resistance to CDDP in our models, as we did not find any alterations in its levels in H460S/R and 41S/R cells; and in the cell line H23, that harbors a nonsense mutation (PTEN_R233*),[36] PTEN levels were undetectable. In fact, although PTEN can be altered by methylation, mutation and homozygous deletion in human tumors, in NSCLC those alterations occur at low frequency.[36,37]

These results indicate that IGFBP-3 methylation induces the phosphorylation of AKT in CDDP-resistant human cancer cell lines. In fact, AKT is responsible in part, of the resistance to the agent in these cells, as *IGFBP-3* overexpression, lowered the AKT phosphorylation levels inducing an increase in sensitivity to CDDP in 41R cells, reaching an intermediate resistant index between 41S and 41R cells. Similarly, the epigenetic reactivation of *IGFBP-3* expression also increased the sensitivity to CDDP in these cells. We also provide further evidence about the direct role of AKT in the acquired resistance to CDDP, as both the inhibition of AKT phosphorylation and the AKT silencing induced an increase in cell mortality and in cell sensitivity to CDDP, resulting in a significant decrease in the IC50 value in response to CDDP in 41R cells.

Those results indicate that AKT phosphorylation has a role in the survival process to CDDP in 41R cells, supporting previous data

that report an activation of the AKT pathway in the resistance to CDDP in lung cancer cells.[38,39] Therefore, these global results provide evidence confirming *IGFBP-3* promoter hypermethylation as an event that mediates the response to CDDP through the activation of AKT.

Then, we assess through which receptor is AKT activated in the acquired resistance to CDDP. Interestingly, we found lower levels of EGF receptor in 41R cells; this effect of CDDP inducing a decrease in EGFR protein expression has been previously reported in MDA-MB-468 cells.[40] Conversely, the IGFIR was activated only in 41R cells, probably because IGFBP-3 is expressed normally in 41S cells, and could be sequestering IGF-I blocking the receptor activation. In fact, IGFI mediates the sustained phosphorylation of AKT, which is essential for a long-term survival in the protection from toxic agents in glial cells.[41] In addition, the IGFIR activation in 41R cells shrank when IGFBP-3 was overexpressed, confirming the role of IGFBP-3 in the AKT activation through IGFIR phosphorylation. We next confirmed the presence of membrane IGFIR and partial receptor internalization after IGFI stimuli in 41S cells, whereas in 41R cells we detected mainly the intracellular IGFIR that strongly increased with IGFI treatment, consistent with receptor internalization.[42] The chronic receptor activation in 41R cells could be mediated by the downregulation of IGFBP-3 expression that causes continuous IGFI cell exposure. This growth factor stimulus could ultimately sustain the Akt phosphorylation observed in these cells through the described IGFIR internalization and recycling process.[41] Although we did not find any changes in the EGFR mRNA levels between 41S and 41R cells, there was a very low EGFR signaling in 41R cells compared with the parental sensitivity, indicating that alterations may be at posttranscriptional level, confirming our data obtained by western blot (WB). These results could explain why several phase III randomized trials combining standard chemotherapeutics with gefitinib, an inhibitor of EGFR tyrosine kinase domain, have failed to show benefits in advanced NSCLC patients.[43,44]

Those results were not specific for the cell lines 41S/41R, as IGFBP-3 overexpression in the lung and pancreatic cancer cell lines H1299 and PANC-1, induced a notable decrease in the IGFIR phosphorylation after CDDP treatment, concomitant with a decrease in pAKT levels; same pattern regarding AKT phosphorylation was also found in the H727 lung cancer cell line. Those results indicate that IGFBP-3 expression regulating the IGFIR/AKT pathway is probably a common mechanism of cell sensitivity to CDDP in different tumor types. In addition, the IGFBP-3 overexpression also induced a severe decrease in cell survival in the four human cancer cell lines tested, which harbor a methylated IGFBP-3 promoter; moreover, there was a correlation between the IC50 to CDDP and the mortality rate in those cells overexpressing IGFBP-3. This result confirms a strong correlation between IGFBP-3 expression and cell mortality regarding sensitivity to CDDP. Explained in terms of translational application, those tumors lacking *IGFBP-3* expression could be suitable for targeted therapy with demethylating agents, as there is a connection between their CDDP resistance and the cell mortality expected after IGFBP-3 reexpression.

We ultimate this study translating to the clinic our laboratory results, in order to gain insight into the cellular pathways involved in the CDDP-acquired resistance process in NSCLC primary tumors. We found predominant activation of IGFIR, but not EGFR in NSCLC patients, being the pEGFR percentage similar to previously reported data.[45] In addition, the activation of IGF-I receptor was a frequent event in NSCLC primary tumors that were resistant to CDDP, and those patients with phosphorylated IGFIR showed concomitant activation of AKT. Those results could explain previous data showing a constitutive AKT phosphorylation through the activation of IGFIR in cells resistant to EGFR inhibition.[46] Furthermore, both *IGFBP-3* promoter methylation and IGFIR/AKT phosphorylation concurs only in CDDP-resistant NSCLC patients, indicating that *IGFBP-3* methylation-derived deficiency could mediate the resistance to CDDP in NSCLC patients though the activation of the IGFIR/AKT pathway. In addition, combination of *IGFBP-3* promoter methylation with IGFIR, and/or AKT, and/or EGFR activation status could be used to predict resistance or sensitivity to CDDP in NSCLC samples. In fact, in this manuscript we present a predictive test that considers that a NSCLC sample needs at least two modifications out of these four: *IGFBP3* methylation, IGFIR phosphorylation, EGFR phosphorylation and AKT phosphorylation, to get a true phenotypic change in terms of CDDP response, with an accuracy and precision of 0.84 and 0.9, respectively, and with a positive likelihood ratio 9.75. Obviously, it is necessary to evaluate this test with additional populations to prove its clinical utility of selecting patients with a high probability to respond to CDDP alone or with a combined therapy based on IGFIR, EGFR and/or AKT inhibitors.

MATERIALS AND METHODS

Cell lines, drugs and treatments

H-460 and H-23 human NSCLC cell lines were purchased from the ATCC (American Type Culture Collection; Manassas, VA, USA), their CDDP-resistant variants H23R and H460R were established previously in our laboratory[16] and cultured as recommended. The CDDP-sensitive and -resistant ovarian cancer cell lines 41M and 41MR, hereafter called 41S and 41R, respectively, were provided by Dr L Kelland and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Additional four human cancer cell lines (H1299, H727, HT-29 and PANC1) were purchased from the ATCC or the ECACC (European Collection of Cell Cultures; Sigma-Aldrich, Madrid, Spain) and cultured as recommended. 5-aza-2′-deoxycytidine and Trichostatin A; Sigma-Aldrich) were used following methodology previously described.[11] CDDP was obtained from Farma-Ferrer (Barcelona, Spain) and the specific PI3K inhibitor LY-294002 was obtained from Calbiochem (Darmstadt, Germany).

For reexpression studies, 41R cells were split to low density and exposed to 5-aza-2′-deoxycytidine (5 μM) and Trichostatin A (300 nM) or to phosphate-buffered saline and ethanol (mock cells) at different CDDP doses, followed by the cell viability analysis to CDDP comparing sensitive vs resistant cell lines before and after the epigenetic treatment, as previously described.[38] Simultaneously, same experimental groups were seeded at 700 000 cells per p60 plate and treated as described above, in order to test the *IGFBP-3* levels by quantitative reverse transcriptase PCR (RT–PCR).

For the PI3K pathway inhibition assay, the 41S and 41R cells were treated 24 h after being seeded, either with the LY-294002 inhibitor or with dimethyl sulfoxide as NC for 1.5 h; then CDDP was added and cell viability measured. Simultaneously, same experimental groups were treated with dimethyl sulfoxide or LY-294002 as described above, in order to test by WB the inhibition of AKT phosphorylation as internal control.

NSCLC clinical samples and data collection

Formalin-fixed paraffin-embedded surgical specimens were obtained from 25 patients who had undergone a complete resection (R0) for a histologically confirmed, early NSCLC. All patients had both a perioperative positron emission tomography-computed tomography scan showing localized disease and a pathological confirmation of stage I or II. In addition, an age of $\geqslant$18 years, intraoperative mediastinal node dissection for reliable mediastinal staging or biopsy of nodes at Node 3, without any evidence of disease were the inclusion criteria. Stage III, any involvement of tracheobronchial angle nodes (station 10), mixed histological features and previous diagnosis of cancer within the last 5 years were the exclusion criteria. Histological slides obtained from each block were reviewed by two expert pathologists to confirm diagnosis and to guarantee at least 90% tumoral content. Follow-up was performed according to the criteria used in the Medical Oncology Division from the H La Paz, including clinical assessments and thorax computer tomography (CT) every 3 months for 2 years and every 6 months thereafter. Clinical, pathological and radiological data were recorded by an independent observer at the H La Paz and blinded for statistical analysis.

DNA and RNA extraction, bisulfite modification, methylation-specific PCR and quantitative RT–PCR

DNA from human cancer cell lines was isolated and bisulfite modified as described,[11] and used to analyze *IGFBP-3* methylation status. Methylation-

specific PCR amplification was performed with specific primers to detect methylated or unmethylated modified DNA according to previous data.[16]

Total RNA from human cancer cell lines was isolated as previously described.[11] Reverse transcription and quantitative RT–PCR analysis were performed as described.[16] Samples were analyzed in triplicate using the StepOnePlus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). Primers and probe for *IGFBP-3* expression analysis were purchased from Applied Biosystems (Hs00181211_m1).

Semiquantitative PCR reactions for *IGFBP-3* were performed as described.[16]

Plasmids and siRNA transfections

For *IGFBP-3* overexpression, cell lines were transfected (Lipofectamine 2000, Invitrogen, Barcelona Spain) with either pCMV5 expression vector containing human IGFBP-3 complimentary DNA (cDNA; Origene, Rockville, MD, USA) or the empty vector (pCMV5) as recommended. Twenty-four hours later, cells were seeded in 24-well dishes for cell viability assays or for cell mortality rate estimation. Simultaneously, same experimental groups were either harvested at same incubation periods to test by RT–PCR IGFBP-3 overexpression or followed a CDDP treatment at different doses for protein levels estimation by WB.

For AKT siRNA assays, 41S and 41R cells (700 000 cells per plate) were transfected with 100 nM *AKT*-siRNA or NC-siRNA (HSS105266, Select-RNAi and 2935-200, RNAi-Negative Control, Invitrogen), according to the manufacturer's directions. For the viability assay, cells were seeded in 24-well dishes at 80 000 cells per well 24 h after transfection with *AKT*-siRNA or siRNA-control, 12 h later cells were treated with CDDP and cell viability was quantified. In parallel, same experimental groups were harvested at 24 and 72 h after siRNA transfection, as a time course to test by WB the preservation of siRNA effects on AKT protein levels.

WB, immunofluorescence and immunohistochemical analysis

Cell lines were cultured at a density of 10^6 cells by 60-mm plate, shifted into medium containing 0.5% fetal bovine serum during 24 h, then stimulated with CDDP with different doses. Whole-cell extracts and WB were performed as described.[47]

For fluorescence microscopy, cells were grown onto coverslips, and 48 h later were treated with EGF at 20 ng/ml (Sigma-Aldrich) or IGF-I at 100 ng/ml (Peprotech, London, UK) for 30 min as stimuli controls, then fixed with paraformaldehyde, permeabilized with Triton X-100 and incubated with secondary antibodies coupled to the fluorescent dye alexa-fluor 488 (Molecular Probes, Barcelona, Spain). Images were acquired on a Confocal Espectral Leica TCS SP5 microscope (Leica, Wetzlar, Germany).

Twenty-five formalin-fixed paraffin-embedded, NSCLC surgical specimens were used for immunohistochemistry analysis. Antigens were retrieved with the Dako buffer citrate pH 9 in a DAKO PT Link for 20–40 min (Dako, Glostrup, Germany). Sections were stained using an automated system DAKO Austostainer. Immunohistochemistry was performed using the Dako Envision Flex kit. Immune reactions were developed with diaminobenzidine and sections were counterstained with Harris' hematoxylin. As NCs, adjacent sections were subjected to same procedure with the antibodies incubated with the appropriate blocking peptide when possible or omitting the primary antibody when peptide was not available. Sections of human breast cancer tissue were used as positive controls.

Antibodies used were anti-AKT (BD Biosciences, San Jose, CA, USA), pAKT-Ser473, PTEN, EGFR, pEGFR-Tyr1068 (1H12, WB), pEGFR-Tyr1068 (D7A5, IHC; Cell Signaling, Danvers, MA, USA), anti-IGFIR, anti-pIGFIR-Tyr1161 (Santa Cruz Biotechnology, Heidelberg, Germany), anti-phospho AKT-pS473 (Dakopatts, Glostrup, Denmark) and anti-α-tubulin (Sigma-Aldrich).

Statistical analysis

We have evaluated our data in terms of accuracy and precision in a binary classification in order to identify or exclude a potential response to CDDP. In this regard, response to CDDP measured by *in vitro* IC_{50} was considered the gold standard (resistant or sensitive). Predicted test value for a tissue sample was considered resistant to CDDP if it had two or more positive results when pAkt or pIGFIR, or pEGFR or *IGFBP-3*-methylation was evaluated. Otherwise, the sample was considered as sensitive. Then we confronted the predicted value to the true condition determined by CDDP IC_{50} to calculate accuracy and precision, likelihood ratios, sensitivity and specificity. Additionally, we used the Fisher's exact test within a 2×2 contingence table to calculate the association between our diagnostic test and the true outcome of analyzed samples in terms of CDDP IC_{50}.

ABBREVIATIONS

ATCC, American Type Culture Collection; BS, bisulfite sequencing; CDDP, cisplatin; DFS, disease-free survival; ECACC, European Collection of Cell Cultures; EGFR, epidermal growth factor receptor; *IGFBP-3*, insulin-like growth factor-binding protein-3; IGFIR, insulin-like growth factor-I receptor; MSP, methylation-specific PCR; NSCLC, non-small cell lung cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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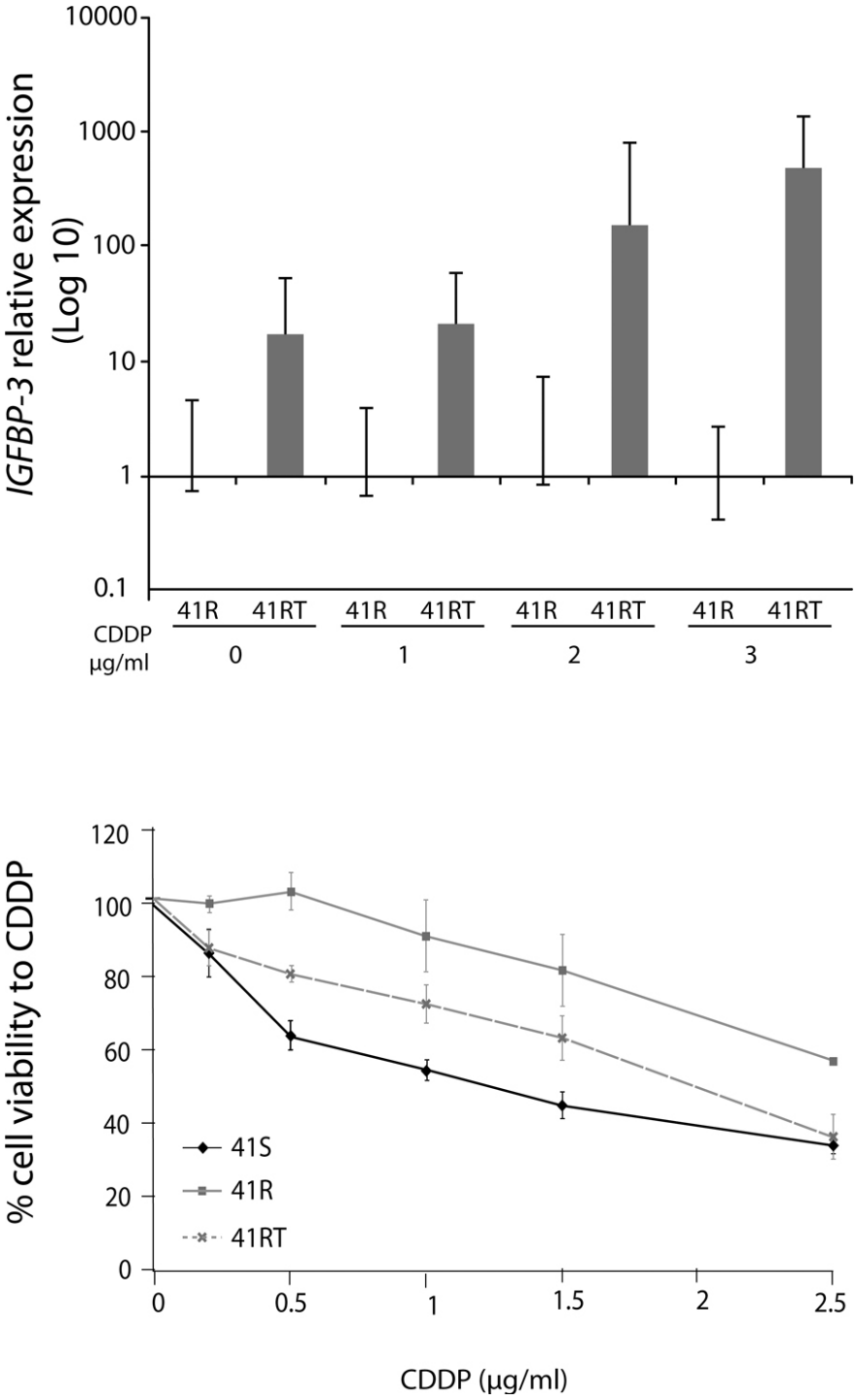
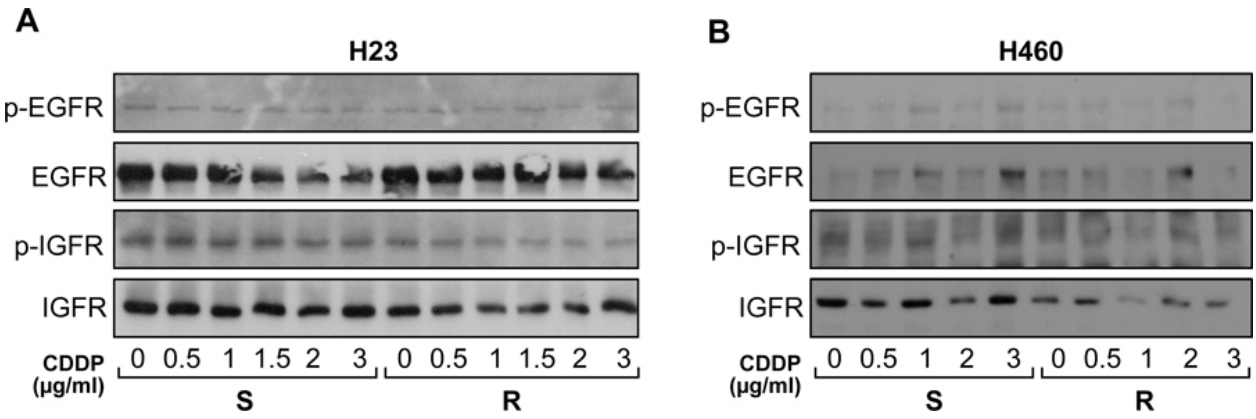
Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Material suplementario

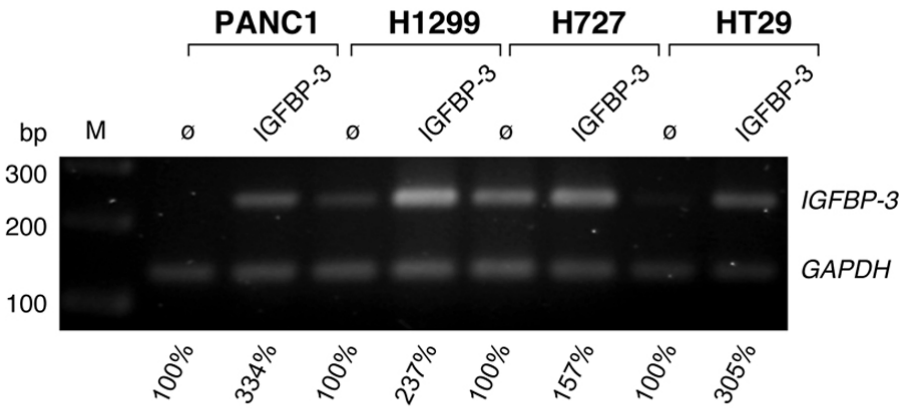


Supplementary Figure 1: Pharmacological reactivation of *IGFBP-3* expression increases cell sensitivity to CDDP treatment. **A)** Combined epigenetic reactivation treatment (RT) with 5-AzadC (5µM), for two cell doubling times and TSA (300nM) for 24h, was effective re-expressing *IGFBP-3* in 41R cells at four CDDP doses tested, measured by qRT-PCR. **B)** Cell viability curve to six different doses of CDDP in 41S, 41R and 41R cells under epigenetic reactivation (41RT). The combined treatment induced an increase in CDDP sensitivity in the resistant 41R cell line, presenting an intermediate response between 41S and 41R cell lines to the CDDP agent.



Supplementary Figure 2: Activation of EGF and IGFI receptors in H23S/R and H460S/R cell lines. A and B) The protein levels of p-EGFR, p-IGFIR, EGFR and IGFIR were measured by WB in H23S, H23R, H460S and H460R cell lines. Cells were seeded and 24 hours later incubated in serum-depleted medium for 24 h and then treated with the indicated CDDP doses for 6 hours. Cell Protein extracts were obtained and 20 µg of total protein was subjected to western blotting; membranes were hybridized with antibodies against p-EGFR, p-IGFR, EGFR and IGFR.

A



B

	IC50 (µg/ml)	Viability Rate ± SD	Mortality Rate (100%)	P-value
HT29+Ø	25	100±6.1	-	
HT29+IGFBP-3		55.3±25.7	44.7	<0.005
H727+Ø	9	100±8.3	-	
H727+IGFBP-3		69.4±25.7	30.6	<0.05
H1299+Ø	6	100±8.6	-	
H1229+IGFBP-3		77.6±20.9	22.3	0.15
PANC1+Ø	4	100±16.7	-	
PANC1+IGFBP-3		71.4±1.3	28.6	0.15

Supplementary Figure 3: The role of *IGFBP-3* mediating CDDP resistance is a frequent process in tumorogenesis. The cell lines PANC-1, H1299, H727 and HT29 represent different types of human cancers, that harbor a methylated promoter for *IGFBP-3* gene and different IC_{50} to CDDP. **A)** The four cell lines were transfected with 1.5µg of human *IGFBP-3* cDNA or the empty vector (Φ), *IGFBP-3* expression was measured by RT-PCR 24 hours after trasfection and *GAPDH* mRNA was co-amplified as a loading control. **B)** Data represent the IC_{50} inhibitory concentration that kills 50% of the cell population 24 h after CDDP exposure in the four cell lines; Cell viability and mortality rates were calculated as the percentage of alive or dead cells observed 24h after *IGFBP-3* trasfection over the same cell line transfected with the empty vector (100%). P<0.05 was considered a significant change (Student's t-test).

Samples	Histo. Type	TNM	Stage	Age	Gender	DFS	CDDP	IC50	*IGFBP-3* MSP	pIGF-IR	pEGFR	pAKT	Test-Prediction
1	Large cell	T2N1	2B	58	M	3	S	0,005	U	NEGATIVE	NEGATIVE	NEGATIVE	S
2	Adeno	T2N1	2B	60	M	60	S	0,005	U	NEGATIVE	NEGATIVE	POSITIVE	S
3	Adeno	T2N1	2B	49	F	12	S	0,006	U	NEGATIVE	NEGATIVE	NEGATIVE	S
4	SCCA	T2N1	2B	58	F	1	S	0,007	U	NEGATIVE	NEGATIVE	NEGATIVE	S
5	SCCA	T3N0	2A	64	M	1	S	0,008	M	NEGATIVE	NEGATIVE	NEGATIVE	S
6	Adeno	T2N1	2B	54	M	1	S	0,008	U	NEGATIVE	NEGATIVE	NEGATIVE	S
7	SCCA	Unknown	Unknown	75	M	1	S	0,01	U	NEGATIVE	NEGATIVE	POSITIVE	S
8	SCCA	T2N0	1B	74	M	72	S	0,01	M	NEGATIVE	NEGATIVE	NEGATIVE	S
9	Large cell	T2N2	3A	58	M	69	S	0,01	U	NEGATIVE	NEGATIVE	NEGATIVE	S
10	Adeno	T3N0	2B	58	M	1	S	0,09	U	NEGATIVE	NEGATIVE	NEGATIVE	S
11	Adeno	T2N0	1B	61	M	62	S	0,5	U	POSITIVE	NEGATIVE	POSITIVE	R*
12	SCCA	T2N1	2B	61	M	1	S	1	U	NEGATIVE	NEGATIVE	NEGATIVE	S
13	Large cell	T2N0	1B	68	M	10	S	5	U	NEGATIVE	NEGATIVE	NEGATIVE	S
14	SCCA	T2N0	1B	65	M	35	R	10	M	NEGATIVE	NEGATIVE	POSITIVE	R
15	SCCA	T2N0	1B	78	M	7	R	14	U	NEGATIVE	POSITIVE	NEGATIVE	S*
16	SCCA	T2N0	1B	77	M	1	R	>100	M	NEGATIVE	NEGATIVE	NEGATIVE	S*
17	Large cell	T2N1	2B	59	M	4	R	>100	M	NEGATIVE	POSITIVE	NEGATIVE	R
18	SCCA	T2N1	2B	58	M	61	R	>100	M	POSITIVE	NEGATIVE	POSITIVE	R
19	Large cell	T1N0	1A	48	M	15	R	>100	U	POSITIVE	NEGATIVE	POSITIVE	R
20	SCCA	T1N0	1A	63	M	16	R	>100	M	POSITIVE	NEGATIVE	POSITIVE	R
21	SCCA	T1N0	1A	65	M	51	R	>100	U	POSITIVE	POSITIVE	NEGATIVE	R
22	SCCA	T1N0	1A	71	M	51	R	>100	M	POSITIVE	NEGATIVE	NEGATIVE	R
23	Adeno	T2N0	1B	67	M	48	R	>100	M	NEGATIVE	NEGATIVE	NEGATIVE	S*
24	SCCA	T3N0	2B	65	M	14	R	>100	U	POSITIVE	POSITIVE	POSITIVE	R
25	Adeno	T1N0	1A	79	M	17	R	>100	U	POSITIVE	NEGATIVE	POSITIVE	R

		Condition as determined by IC50		
		Resistant	Sensitive	
Test prediction	Resistant	9	1*	0.9 (PPV)
	Sensitive	3*	12	0.8 (NPV)
		0.75	0.925	

Supplementary Table 1: Clinicopathological parameters: stages, age, gender, desease free survival (DFS), CDDP response, hypermethylation of *IGFBP-3* and activation of IGFIR, EGFR and AKT proteins data were recorded from 25 NSCLC patients. Test-prediction line described the Predicted test values for a tissue sample. It was considered resistant to cisplatin if it had two or more positive results when p-Akt or p-IGFIR or p-EGFR or m*IGFBP-3* were evaluated. Lower panel confronted the predicted value from our diagnostic test and the true outcome of analyzed samples in terms of cisplatin IC_{50} by using the Fisher´s exact test within a 2x2 contingence table.

NOTE. Age, years; Histo type SCCA, squamous cell carcinoma; Grade: American Joint Committee on Cancer; Stage: American Joint Committee on Cancer stage grouping; CDDP; R, resistant to CDDP, S sensitive to CDDP; IC_{50}, drug concentration that kills 50% of cell population after 72h of CDDP expousure. MSP; M methylated; U, unmethylated for *IGFBP-3*.* indicate the four samples in which our prediction test failed.

Capítulo 3

MKP1 repression is required for the chemosensitizing effects of NFκB and PI3K inhibitors to cisplatin in non-small cell lung cancer.

En resultados previos de nuestro laboratorio se había observado que la fosfatasa dual MKP1 inhibía la apoptosis inducida por CDDP en líneas celulares humanas de CNMP y que además estaba sobreexpresada en células tumorales de pacientes con esta enfermedad en estadios I-II. En este trabajo nuestro objetivo fue estudiar qué otras vías de señalización son importantes en la respuesta a CDDP en líneas celulares de CNMP. Para ello se utilizaron las células tumorales humanas de cáncer de pulmón H460, H1299, H727, A549 y H23, de las cuales H1299 y H727 son 5 veces más resistentes a CDDP que las demás. Se trataron las líneas celulares con CDDP a distintos tiempos y se estudió la activación de diferentes vías de señalización de supervivencia y de apoptosis, así encontramos importantes diferencias en el comportamiento bioquímico de estas células en respuesta a CDDP. Aunque en las cinco líneas celulares el CDDP inducía la activación de JNK y p38, en las células H1299 y H23 apreciamos una activación temprana de dichas quinasas, lo que correlacionaba con los niveles de expresión bajos de la fosfatasa MKP1 en estas dos líneas celulares. Por otra parte las células H1299 y H727 presentaban niveles altos de fosforilación de AKT en condiciones basales, activación que se mantenía a lo largo del tratamiento.

Posteriormente se procedió al estudio en las 5 líneas celulares de los factores de transcripción NFκB, que tienen gran implicación en el crecimiento celular y en supervivencia en tumores humanos. Observamos mediante el estudio tanto de los niveles en el núcleo como de la actividad de estos factores de transcripción, que esta vía de señalización parecía tener una gran importancia en las líneas tumorales H23, H727 y H1299. Una vez recopilados estos datos se eligieron las líneas celulares H460, H1299 y H727, debido a las diferencias que presentaban en cuanto a los niveles de MKP1 y a la activación de las vías de AKT y NFκB, estudiándose el efecto de la inhibición de las vías de PI3K/AKT y NFκB en la respuesta a CDDP. Para ello se utilizaron los inhibidores de estas rutas, LY29402 y BAY11-7082 respectivamente. Así se observó, que en la línea tumoral H1299 tanto la inhibición de la vía de AKT como de la vía de NFκB inducía una sensibilización de las células al CDDP, fenómeno que no ocurría en las líneas celulares H727 y H460, en las que la inhibición de ambas rutas no mostraban o mostraban un incremento muy pequeño de la sensibilidad a CDDP. Debido a que estas dos últimas líneas presentaban niveles altos de expresión de MKP1, se utilizaron las líneas celulares H460cri, H1299cri y H727cri, que tenían inhibida la expresión de MKP1 mediante un ARN de interferencia, para realizar un ensayo de viabilidad como el descrito anteriormente, inhibiendo las vías de PI3K y NFκB y midiendo la respuesta a CDDP. La línea celular H1299 no expresaba MKP1 por lo que se utilizó como control negativo. Esta vez se observó un incremento en la sensibilidad a CDDP en las líneas celulares H460cri y H727cri tratadas con los inhibidores. Sin embargo, no se observaron diferencias en la sensibilidad a CDDP entre las células H1299 y H1299cri, tal como esperábamos. Estos datos indican que la regulación de la expresión de MKP1 en células de CNMP juega un papel fundamental en la respuesta a CDDP cuando se utilizan inhibidores de las vías de NFκB y PI3K/AKT. Finalmente para completar el estudio se analizó el papel de NFκB mediante inmunohistoquímica evaluando la expresión de p65/RelA y RelB en 26 muestras de pacientes de CNMP. Se observó que la presencia de p65/RelA en el núcleo se detecta en un 46% de las muestras de pacientes, mientras que RelB se detecta en un 19%; además las muestras que eran positivas para RelB también lo eran para p65/RelA. Por otro lado, la localización de MKP1 en el núcleo aparece en un 80% de las muestras. También se observó que un 93% de las

muestras que mostraban p65/RelA o RelB nuclear también presentaban expresión nuclear de MKP1, es decir que un 53% de las muestras examinadas presentaban una situación parecida a la línea celular H727 (carcinoma, que presenta p65/RelA y MKP1 en el núcleo), mientras que un pequeño porcentaje (6%) mostraba exclusivamente p65/RelA y RelB activos, como las células H1299 (carcinoma, sólo tinción en núcleo de p65/RelA y RelB), y el resto eran positivas sólo para MKP1 (46,6%), como las células H460 (carcinoma, tinción exclusiva de MKP1 en núcleo). A partir de estos resultados sobre la co-expresión de p65/RelA y RelB, se decidió ensayar el efecto del tratamiento con bortezomib (un inhibidor del proteasoma) en las células de CNMP, y se comprobó en primer lugar que este quimioterápico inhibía la activación de NFκB en estas líneas celulares. Finalmente se estudió el efecto de bortezomib sobre la viabilidad celular en las distintas líneas celulares de CNMP, obteniéndose como resultado que la actividad citotóxica del bortezomib correlacionaba con la actividad basal de NFκB, es decir que las células que tienen una mayor actividad basal de NFκB, como H23 y H1299, son más sensibles a bortezomib. Con todo ello, lo que demostramos en este estudio, es que la activación de PI3K/AKT y NFκB lleva a una reducción de la sensibilidad a CDDP en CNMP, y que la inhibición de estas vías de señalización aumenta la citotoxicidad del CDDP en células de CNMP con niveles bajos de MKP1. Por todo esto, un análisis inmunohistoquímico para detectar la activación de las vías descritas en este trabajo a partir de la muestra de un paciente de CNMP podría indicar la posible respuesta a CDDP o a otros quimioterápicos, como por ejemplo al bortezomib.

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MKP1 repression is required for the chemosensitizing effects of NF-κB and PI3K inhibitors to cisplatin in non-small cell lung cancer

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ABSTRACT

Treatment of non-small cell lung cancer (NSCLC) with cisplatin has a level of antitumor activity still modest. We have shown previously that MKP1/DUSP1 inhibits cisplatin-induced apoptosis in NSCLC cells and is overexpressed in tumors from most patients with stage I–II NSCLC. Here, using different NSCLC cell lines we found that MKP1 and NF-κB are differentially expressed. We studied whether targeting MKP1, NF-κB or both affects cisplatin-induced cell death. MKP1 is expressed in H460 and H727 cells. H727 and H1299 cells showed constitutive phosphorylation of Akt and increased NF-κB activity than did H460 cells. H460-MKP1-siRNA-expressing cells (but not H727-MKP1-siRNA or H1299-MKP1-siRNA cells) exhibit a marked increase in cisplatin response compared with parental cells. Treatment with the PI3K inhibitor LY294002 or the NF-κB inhibitor BAY11-7082 enhanced cisplatin antitumor activity in parental H1299 cells but only weakly affected responses of H727 and H460 cells. MKP1-siRNA expression enhanced the chemosensitization effect of LY294002 and BAY11-7082 on H727 and H460 cells. Additionally, NSCLC cell lines with higher NF-κB-constitutive activation were the most sensitive to PS-341 (Bortezomib), a non-specific NF-κB inhibitor. This finding suggests the proteasome as a suitable strategy in treating NSCLC tumors with high constitutive NF-κB activity. Altogether, these results showed that either an activated PI3K/Akt/NF-κB pathway and/or high MKP1 was linked to reduced sensitivity to cisplatin in NSCLC cells. Inhibition of NF-κB or PI3K potently enhanced cisplatin cytotoxicity in cells with endogenous or genetically induced low MKP1 levels. These findings support the potential improvement in cisplatin responses by co-targeting NF-κB or Akt and MKP1.

1. Introduction

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer. Systemic treatment of advanced/metastatic NSCLC and, more recently, of high-risk surgically

Abbreviations: CDDP, cisplatin; TNF-α, tumor necrosis factor alpha.

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treated patients, involves mainly the use of cisplatin-based chemotherapy. Cisplatin is a DNA-interactive agent that induces lesions in the DNA by forming monoadducts and intra- and interstrand cross-links [1]. Cell death is then mediated by inducing a sustained activation of *N*-terminal c-Jun kinase (JNK) and p38 kinase [2–4]. Unfortunately, the clinical efficacy of cisplatin in NSCLC patients is still far from optimal. This may be related, at least in part, to the presence of various survival pathways in malignant cells that limit or counteract platinum antitumor effects. Upon cisplatin exposure, tumor cells activate survival pathways, including those mediated by dual specific phosphatase MKP1 (DUSP1) [2–4], PI3K [5] and the transcription factor nuclear-factor kappaB (NF-κB) [6]. Gaining further knowledge on the role of these pathways in chemoresistance should lead to novel strategies to enhance cisplatin activity.

MKP1 is an immediate early gene regulated at the transcriptional level by mitogenic, inflammatory, and DNA-damaging stimuli [3,7,8]. Dephosphorylation and inactivation of JNK by MKP1 protects against cisplatin-induced apoptosis [2]. With regards to NSCLC, we have reported that MKP1 plays an important function in NSCLC tumor growth and in response to cisplatin treatment [9]. NSCLC cells expressing a small interfering RNA (siRNA) of MKP1 were more sensitive to cisplatin and grew more slowly when injected into nude mice. Chemical compounds that inhibit, non-specifically, MKP1 expression also induced higher sensitivity to cisplatin. Furthermore, these effects of MKP1 were very specific because inhibition or overexpression of an MKP1-related phosphatase, MKP2, did not alter tumor growth or response to cisplatin in NSCLC cells [9]. These results suggested MKP1 as a novel target for tumor regression and, particularly, for chemosensitizing NSCLC to cisplatin. Since high expression of MKP1 has been reported in NSCLC [7] MKP1 may have implications to improve lung cancer treatment strategies. In addition to MKP1, both PI3K/Akt and NF-κB survival pathways can also limit cisplatin antitumor effects in lung cancer [5]. Further characterization of these pathways is needed, taking in consideration that inhibitors of PI3K and NF-κB are in extensive preclinical and early clinical development.

Class I PI3K comprises a family of heterodimeric complexes, each composed of a p110 catalytic subunit and an adaptor subunit that exists predominantly as p85 [10,11]. PI3K phosphorylates PI(4)P and PI(4,5)P to produce PI(3,4)P2 and PI(3,4,5)P3, which recruit proteins such as Akt/protein kinase B [10] to the cytoplasmic membrane. In turn, Akt inactivates pro-apoptotic proteins such as BAD and caspase-9, thus promoting cell survival [12–14]. With regards to NSCLC, increased gene copy number of PI3K catalytic subunit α and phosphorylated Akt expression has been observed in clinical specimens. In addition, inhibition of PI3K/Akt by pharmacological or genetic approaches reduced proliferation in some NSCLC cancer cell lines [15–18].

NF-κB comprises a family of inducible transcription factors that, among many other roles, protect cells from apoptosis induced by several chemotherapeutic agents [6,19]. In unstimulated cells, NF-κB forms cytoplasmic dimmers associated with a family of inhibitory molecules known as IκBs [20,21]. There are two known NF-κB pathways [22]. In the canonical pathway, activation of NF-κB involves the phosphorylation of IκBs through the IκB kinase signalosome complex [23–25]. This is followed by rapid ubiquitin-dependent degradation by the 26S proteasome. This allows NF-κB dimmers, mainly p65/p50, to translocate to the nucleus where they stimulate expression of target genes. In the non-canonical pathway, the p100-RelB complex is activated by an IκKα homodimer [26]. Notably, cisplatin activates NF-κB through MEKK1, and this activation is modulated by c-Jun, the main substrate of the JNK pathway [27]. This effect, again, can limit cisplatin-induced cell death. Many current antitumor therapies seek to block NF-κB activity as a means of inhibiting tumor growth or sensitizing the tumor cells to chemotherapy [28]. Bortezomib (PS-341, Velcade™) is a potent and selective inhibitor of the chymotryptic activity of the 20S proteasome with a cytotoxic activity in several malignant cell lines. It has clinically proven efficacy in patients with multiple myeloma and malignant lymphoma [29]. The earliest mechanism of action attributed to Bortezomib was the inhibition of NF-κB signaling by stabilizing IκB. Besides, proteasome inhibitors have a number of additional mechanisms of action beyond NF-κB inhibition, due to the effect on proteolysis such as stabilization of pro-apoptotic proteins as p53, Bax, while reducing levels of some antiapoptotic proteins such as Bcl2.

Here, we have examined the relative role of MKP1, PI3K/Akt and NF-κB survival pathways in preventing cisplatin-induced cell death. Our data revealed that MKP1 played a key role in determining the ability of PI3K and NF-κB inhibitors to sensitize NSCLC cells to cisplatin cytotoxicity. In cells with undetected MKP1 expression, inhibition of either PI3K or NF-κB was sufficient to enhance cisplatin effects while in cell lines with detectable expression of MKP1 this was not observed. We then addressed whether MKP1 expression was a marker or a cause of these differential chemosensitizing effects. To this end, NSCLC cell lines were interfered for MKP1 expression with siRNA. Downmodulation of MKP1 expression resulted in a dramatic enhancement of the chemosensitizing effects of both PI3K and NF-κB inhibitors in NSCLC. The data strongly support that MKP1 and PI3K, or MKP1 and NF-κB, signaling pathways contribute independently to cisplatin resistance in NSCLC cells. A concerted inhibition of these pathways emerges as a novel strategy to improve cisplatin antitumor activity.

2. Materials and methods

2.1. Cell culture, antibodies, and reagents

H460, H727, H1299, H23 and A549 cell lines were purchased from the American Type Culture Collection, maintained in RPMI supplemented with 10% fetal-bovine serum. Antibodies used were as follows; unless indicated otherwise, these reagents were from Santa Cruz Biotechnologies, Santa Cruz, CA: anti-MKP1 (M18,), anti-pJNK (V7391, Promega, Madison, WI), anti-JNK1 (C-17), anti-p38 (C20), anti-relB (C19), anti-pP38 (9211S, Cell Signaling

Technology, Beverly, MA), anti-β-tubulin (T9026, Sigma–Aldrich, St. Louis, MO), anti-c-Jun (H16), anti-p65/relA (H286). The enhanced chemiluminescent kit was from Amersham (Amersham, UK). Cisplatin was purchased from Calbiochem. Puromycin and geneticin were from Sigma–Aldrich.

2.2. Plasmids and constructions

(−453/+80) HIV-Luc contains the NF-κB-binding sites of the HIV promoter followed by the luciferase expression cassette [28]. Gal4-p65TAD1 was obtained from Lienhard Schmitz [30,31]. pRetroSuper was from OligoEngine.

The MKP1pRetroSuper derived vector (pRS) has been described [7]. Ampicillin-resistant colonies were selected and checked by sequencing using pRetroSuper vector sequence primers 5′ACCTCCTCGTTCGACGG-3′ for direct and 5′TGTGAGGGACAGGGGAG-3′ for reverse sequencing.

2.3. Transfection and analysis of gene expression

For the transient transfection assays, cell lines and derived siRNA clones were transfected with Lipofectamine-2000 (Invitrogen). The maximal total amount of DNA used was 0.5 μg in a six-well dish. pCMVrenilla plasmid was cotransfected along with the HIV-Luc for transfection efficiency control. For stable transfections, 1 μg of the siRNA-derived construct per million cells was transfected into 80–90% confluent dishes. Mock transfections were also carried out using Lipofectamine-2000 but no siRNA. Cells were treated with 2.5-(H727 cells) or 5 μg/ml-(H1299 cells) of puromycin for 24–48 h and kept for selection. Stable transfection was confirmed by western blotting. Luciferase and renilla assays were performed according to the manufacturer's instructions (Promega) as described previously [30]. Transfection efficiencies were corrected by cotransfection of pCMVrenilla and by measuring renilla activity. Each assay was performed in triplicate in each experiment, and each experiment was repeated three times.

2.4. Cell extracts and western blots

Whole cell and nuclear extracts were prepared essentially as described previously [3,30]. Western blotting was done by standard methods [3].

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (2 μg of protein) of cells were incubated with a ^{32}P-labeled probe containing the NF-κB-binding site [30]. The protein–DNA complexes formed were analyzed by EMSA as previously described [27]. Specific complexes were identified by incubating, nuclear extracts with the corresponding antibody. In order to correct for protein each band shift we have used a probe to the transcription factor Oct-1. Specific competition was performed by incubation with 10-fold higher amount of the NF-κB probe.

2.6. Cell viability determination

Cell viability was determined using a crystal violet staining method followed by colorimetric assay as described [3].

2.7. Proteasome activity assay

Cell lysates were prepared and the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr, AMC was used according with the procedures described by the 20S proteasome activity Assay kit (Chemicon, Temecula, CA). Free AMC liberated by the substrate hydrolysis was quantified for 90 min at 1-min intervals on a microtiter plate fluorometer (FLUOstar Optima, BMG Labtech, Durham, NC, excitation 355 nm, emission 460 nm). The data were plotted as arbitrary fluorescence units and proteasome activity values (% control) were obtained.

3. Results

3.1. Activation of JNK and Akt signaling pathways in NSCLC cell lines

To understand better the cellular responses evoked by cisplatin treatment in NSCLC cells, we used five different cell lines H460, H1299, H727, H23 and A549. Cell lines H727 and H1299 were about 5-fold more resistant to cisplatin than were H460, H23 and A549 cells (data not shown). We assayed for the expression of total and phosphorylated forms of JNK, p38 and in the expression of MKP1. We chose these proteins since we have reported previously that JNK activation is involved in cisplatin-induced cell death in NSCLC [3,4], p38 has been also linked as a mediator of cisplatin cell death, and on another hand, MKP1 is able to deactivate both JNK and p38. Activation of JNK occurred in all the cell lines but, of note, with different kinetics. JNK activation kinetics was slow and late in H727 cells, basal levels were detected in H23 and H1299 cells, and a similar kinetic was detected in H460, H1299, A549 and H23 increasing at 6 h up to 24 h. Only H23 cells showed a more transient activation due to increased apoptosis at 24 h. p38 kinase was activated early in H1299 and H23 cells, but the kinetics of activation were late in H460 and almost undetected in H727 and A549 cells. H460, A549 and H727 cells expressed basal levels of MKP1, which decreased after cisplatin treatment, when activation of both JNK and p38 was detected. Low levels of MKP1 were detected in H23 cells and H1299 did not express detectable levels of MKP1 protein (Fig. 1A) or mRNA (data not shown). This observation agrees with the earlier kinetic of activation observed for p38 and JNK and with the basal levels of JNK phosphorylation observed in both cell lines.

We also checked for Akt phosphorylation, another signaling pathway that is altered frequently in NSCLC. H460, H23 and H727 cells showed increased phosphorylation of Akt in response to cisplatin (Fig. 1B). We observe a very transient activation of Akt in A549 cells. In contrast, H1299 cells exhibited an earlier and more transient kinetic of activation for pAkt than did the other cell lines, which showed even higher basal and induced levels and prolonged phosphorylation of Akt. This pattern of Akt phosphorylation agrees with the expression of *PTEN* in these cell lines. *PTEN* was highly expressed in H460 cells (Fig. 1C), almost undetectable in H1299, and low in H727, H23 and A549 cells.

3.2. Nuclear protein levels of NF-κB family of transcription factors in NSCLC cell lines

NF-κB is a transcription factor involved in cell growth and survival in human tumors. We first studied the nuclear levels of NF-κB family members, both from the canonical and non-canonical NF-κB activation pathway. We investigated the levels of two transcriptionally active members of NF-κB, p65/relA and relB. p65/relA nuclear levels were higher in H1299, H23 and A549 cells, lower in H727 cells and undetectable in H460 cells (Fig. 2A). relB levels were very high in H1299 cells (Fig. 2A), lower in H23 and A549 cells and undetectable in H727 and H460 cells.

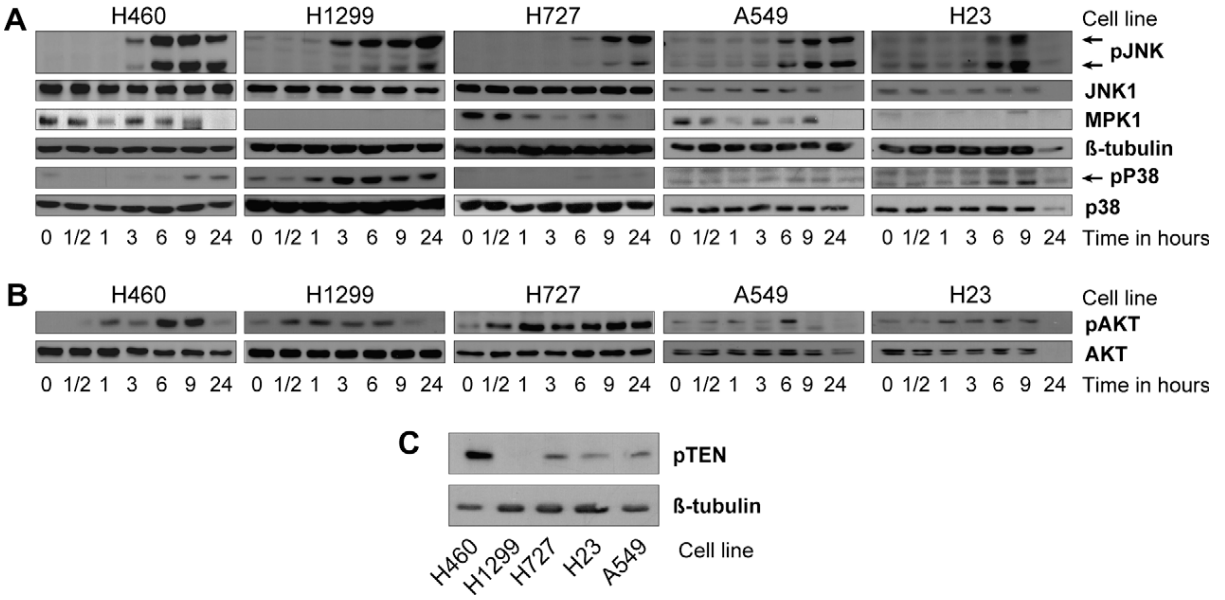

Fig. 1. Kinetics of activation of JNK, p38, and Akt in H460, H1299, H727, A549 and H23 cells treated with cisplatin. H460, H1299, H727, A549 and H23 cells were seeded and then incubated in serum-depleted medium for 16 h, after which 10 μg/ml cisplatin was added for the indicated times. Protein extracts of cells were obtained at each time, and 20 μg of total protein was subjected to western blotting and the membrane was hybridized with antibodies against (A) pJNK, JNK, pP38, p38, β-tubulin and MKP1 and (B) pAkt and Akt. (C) Basal levels of *PTEN* were detected in the three cell lines. The loading control of total protein was checked using an antibody against β-tubulin.

In total extracts, basal levels of both p100 and p52, two non-transcriptionally active members, increased dramatically in H1299 cells, whereas the levels p105 or p50 subunits did not differ between the cell lines (data not shown).

We further investigated the levels transcriptionally active complexes at the NF-κB by performing electrophoretic mobility assays. We have found that H1299 and H23 cells showed higher proportion of NF-κB complexes, when compared with H727 (Fig. 2B) which agrees with the results obtained above (Fig. 2A). In order to characterize which κB proteins were bound constitutively to the NF-κB sites, we use specific antibodies of p65, relB and p50 to compete binding in nuclear extracts form H1299, H23 and H727 cells. The results indicated that H1299 cell complexes comprise p65, relB and p50 (Fig. 2C) proteins while in H23 and H727 κB complexes were composed mainly by p65/p50 heterodimers. The results indicate that in NSCLC cell lines both NF-κB signaling pathways, canonical and non-canonical, contribute to NF-κB signaling.

To investigate whether levels of nuclear NF-κB proteins correlated with the magnitude of NF-κB-dependent transcription, we used the HIV-Luc reporter, which contains two κB-binding sites (Fig. 2D). Although H460 cells showed almost no basal activation of the NF-κB reporter, H23 cells showed the highest increase in basal NF-κB-dependent transcription, followed by H1299 cells, H727 and A549 cells in a decreasing order. These differences were not due to changes in transfection efficiencies, since all luciferase values were normalized to that of an internal renilla control. Different cellular stimuli can activate NF-κB transcription by mechanisms independent of its nuclear translocation [31]. These alternative mechanisms involve stimulation of the transactivation domain of both the basal and induced levels of the p65 subunit of NF-κB. Therefore, we studied whether the differences observed were dependent on the transcriptional activation of p65. To address this question, we used a plasmid encoding the Gal4–p65 fusion protein, where the sequences encoding the DNA binding domain of Gal4 have been joined with sequences encoding the TAD1 of p65 [31]. Once transfected with the Gal4-Luc reporter, this construction allowed us to determine whether the cellular signals activated in H1299, H23, A549 and H727 cell lines regulate gene expression by specifically targeting TAD1 of the p65/relA protein. The results showed that basal activation of the p65 TAD1 was higher in H727 cells (Fig. 2E) and much lower in the other cell lines. These results indicate that, in H727 cells, basal NF-κB transcriptional activity is partially related to an increase in p65 transactivation potential.

3.3. Inhibition of PI3K or NF-κB sensitize NSCLC cells expressing low MKP1 levels towards cisplatin

For combinatorial and mechanistic experiments, we chose three cell lines (H1299, H727 and H460) from our panel, due to their differential basal and cisplatin-induced expression of PI3K and NF-κB pathways and MKP1 (see Fig. 1), as well as for their good transfectability with siRNA (see below). We could not obtained MKP1-siRNA-derived viable cell lines from H23 or A549 cells. We first studied the effects of PI3K inhibition due to its involvement in the control of survival and proliferation of cancer cells [15]. We used the PI3K inhibitor LY294002 to inhibit the signaling pathway triggered by PI3K, and we checked the timing of LY29402 inhibition of phosphorylation of Akt in H1299 cells. Cells were pretreated with LY29402 for 4 h and then stimulated with either cisplatin (Fig. 3A) or epidermal growth factor (EGF) (Fig. 3B). Treatment of cells with LY29402 inhibited phosphorylation of Akt in response to both stimuli (Fig. 3A and B). We next tested whether treatment of parental cell lines with LY29402 (10 μM) influenced the cell survival in response to cisplatin treatment (Fig. 3C). LY29402-pretreated H727 and H460 cells, both expressing MKP1, showed no or very little increase in sensitivity to cisplatin. This contrasted with the data obtained in the western blots, where we detected an important activation of Akt in H727 cells, which is usually a consequence of PI3K activity (Fig. 1B). On the other hand, H1299 cells (with undetected MKP1 expression) were more sensitive to the inhibitor and the IC_{50} values showed a difference of nearly two orders of magnitude in response to cisplatin (Fig. 3C).

In addition to PI3K, activation of NF-κB may represent another mechanism responsible for de novo and inducible chemoresistance because of its antiapoptotic action [15]. Both H1299 and H727 cells showed constitutive activation of the NF-κB reporter construct (Fig. 2D and E).

We next investigated the effect of canonical NF-κB inhibition in combination with cisplatin treatment. To this end, we used Bay11-7082, which acts at the level of IκB by inhibiting its phosphorylation and consequently the translocation of p65-NF-κB to the nucleus. We first tested the effect of pretreatment with Bay11-7082 on accumulation of NF-κB complexes in the nuclei. H1299 cells were treated for 4 h and the levels of κB complexes were detected by band shift assays. Fig. 4A shows a marked decrease in the level of κB complexes in H1299 cells after treatment with Bay11-7082.

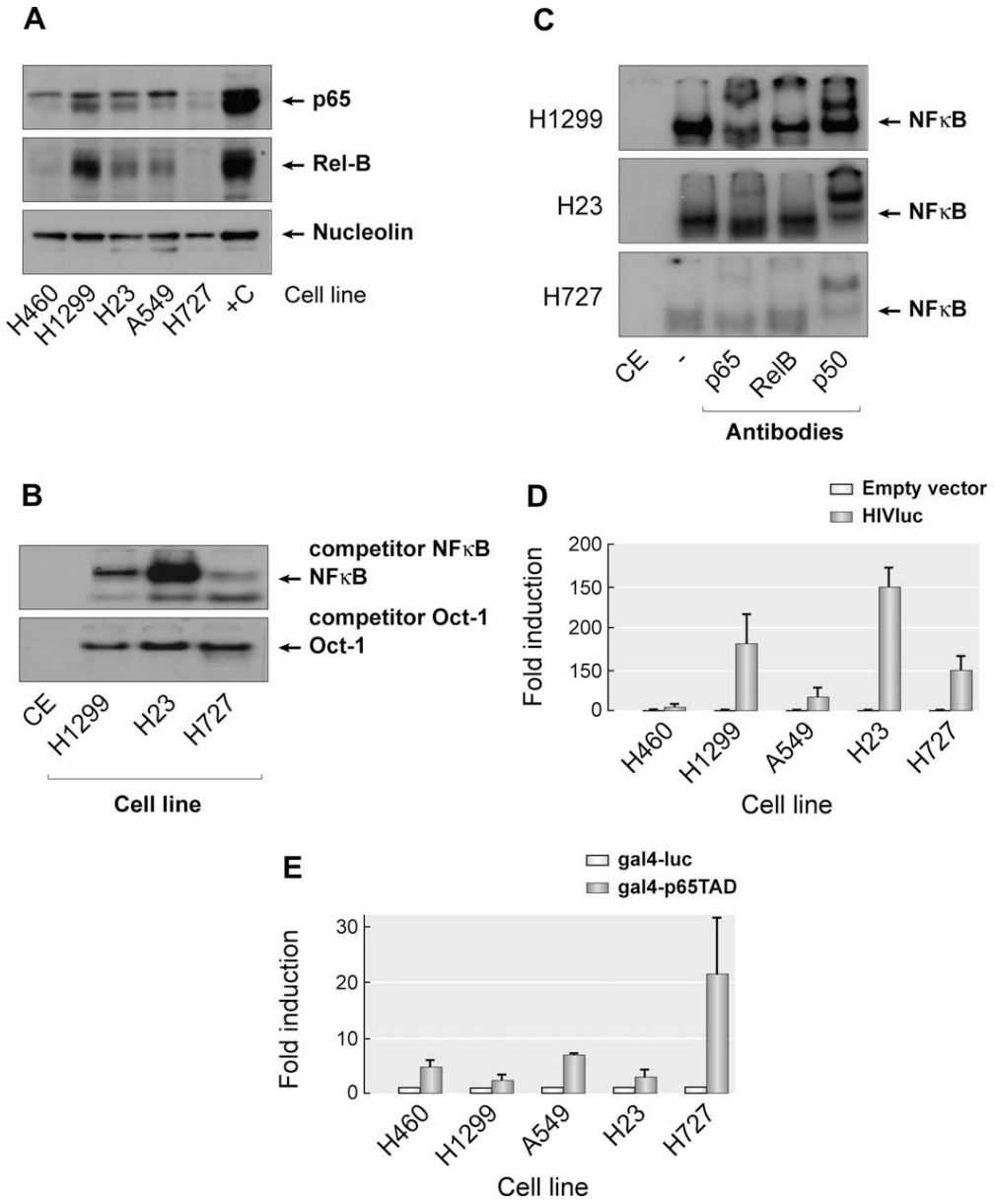

Fig. 2. Nuclear levels of NF-κB proteins in NSCLC cells. (A) H460, H1299, H727, A549 and H23 cells were seeded and incubated in serum-depleted medium for 16 h. Nuclear protein extracts of cells were obtained, and 5 μg of nuclear protein was subjected to western blotting and the membrane was hybridized with antibodies against p65/relA and relB, an antibody against nucleolin was used as the loading control for nuclear protein, control cells stimulated with TNF-α. (B) EMSA analysis of NFkB binding. Band shift assays were performed with nuclear extracts obtained in B and using 5 μg of protein. Specific competition was obtained with the NFkB cold probe (upper panel). An Oct-1 probe was used for loading control (lower panel). (C) Identification of kB complexes in H1299, H23 and H727 cells. Nuclear extracts obtained in A were subjected to EMSA assay and binding to the kB oligonucleotide was competed with specific antibodies towards, p65/relA, p50 and relB. Specific (NFkB) competitor is indicated in the figure (c). (D) NF-κB-dependent transcription in NSCLC cells. H460, H1299, H727, A549 and H23 cells were transfected by Lipofectamine either with empty vector or with 0.5 μg of (−453/−80) HIV-luc per 60-mm well. The fold increase in luciferase activity was calculated relative to the value for the empty vector-transfected cells. (E) H460, H1299, H727, A549 and H23 cells were cotransfected with a 5X-Gal-luc reporter plasmid (0.25 μg) or 5X-Gal-luc reporter plus the expression vector encoding GAL4-p65 TAD1 (0.25 μg). After 24 h, the cells were collected and the relative luciferase activity was determined as in D. Data shown in this figure represent the mean of a single experiment performed in triplicate ±SD and are representative of three or more experiments with similar results.

One set of cells was treated with Bay11-7082 (5 μM) for 4 h before adding increasing concentrations of cisplatin, and another set of non-pretreated cells was treated with cisplatin in an identical manner. In both H727 and H460 cell lines, the sensitivity to cisplatin did not increase when pretreated with Bay11-7082 (Fig. 4B). In contrast, H1299 cells were very sensitive to this inhibitor and showed a difference of nearly two orders of magnitude in response to cisplatin (Fig. 4B). Notably, these results were very similar to what we observed when we used a PI3K inhibitor (see above) instead of Bay11-7082. Both experiments pointed to the presence of MKP1 as a limiting factor in the chemosensitizing effects of both PI3K as well as specific, canonical, NF-κB inhibitors towards cisplatin.

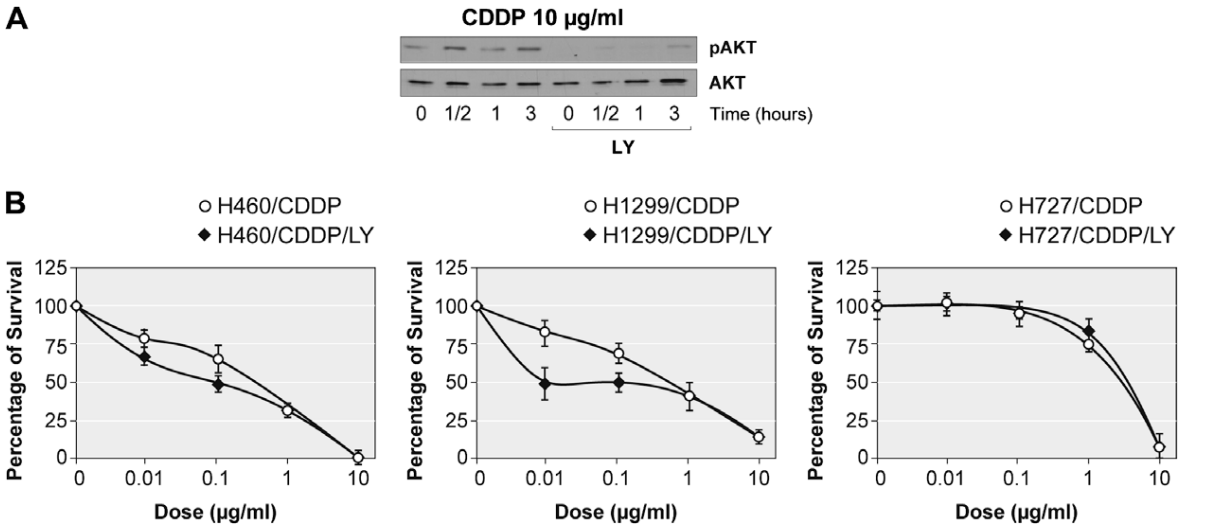

Fig. 3. Effect of pretreatment with the PI3K inhibitor LY294002 on cisplatin survival in NSCLC cells. (A) Treatment of LY294002 in H1299 cells. H1299 cells were seeded and then incubated in serum-depleted medium for 16 h. Cells were treated either with cisplatin (10 µg/ml) or EGF (20 ng/ml). The cells had been pretreated with LY294002 protein extracts of cells were obtained at each time, and 20 µg of total protein was subjected to western blotting and the membrane was hybridized with antibodies against pAkt or Akt as indicated. (B) Cell viability in response to cisplatin (at the indicated concentrations) of H460 pSuperRetro (H460), H1299 pSuperRetro (H1299), and H727 pSuperRetro (H727) cells and the effect of LY294002. When indicated (LY), cells were pretreated for 4 h with LY294002 (10 µM). Data represent the means of two experiments performed in quadruplicate.

3.4. Inhibition of MKP1 expression in NSCLC, facilitates chemosensitization of PI3K and NF-κB inhibitors to cisplatin

Since evidence obtained in our laboratory strongly suggested that expression of MKP1 in NSCLC cell lines protected against PI3K and NF-κB sensitization towards cisplatin-induced cell death (9), we generated MKP1 siRNA-derived cell lines from H1299(H1299cri) and H727(H727cri) cells. The generation and expression of MKP1 in H460MKP1-siRNA cells has been described elsewhere (H460cri) by us [9]. H1299 did not express MKP1 (Fig. 1A), but were transfected for experimental control. The expression of MKP1 protein in H727 cells was inhibited by 30% in the resulting cell line (Fig. 5A). We next studied the effect of LY294002 in MKP1-siRNA-derived cell lines. This time, we observed a dramatic increase in sensitivity to cisplatin in both H460cri and H727cri cells, which have lower MKP1 levels than their parental cells (Fig. 5B). In H1299 cells, that endogenously lack MKP1, the level of enhancement of cytotoxicity did not differ between wild-type and H1299cri cells. These results suggest that LY294002 has a direct sensitization effect in cells that express pAkt but low MKP1. In contrast, the basal expression of MKP1 is able to interference with this sensitization. The increased sensitivity to cisplatin of MKP1 SIRNA-derived H460 and H727 cell lines treated with LY294002 is not due to an increase in phosphorylation of JNK (data not shown), more likely to the inhibition of PI3K survival pathway.

We next investigated the effect of Bay11-7082 pretreatment on MKP1-siRNA-derived cells (Fig. 5C) This time, we found very strong sensitization of H460cri and H727cri cells, as shown by a decrease of the IC_{50} to <0.01 µg/ml cisplatin. The increased sensitivity to cisplatin of MKP1-SIRNA derived H460 and H727 cell lines treated with Bay11-7082, is not due to an increase in phosphorylation of JNK (data not shown), more likely to the inhibition of NF-κB survival pathway, since activation of NF-κB by cisplatin is inhibited in these cells (data not shown). The IC_{50} of H1299cri did not change considerably from the values obtained with the wild-type, implying that no further sensitization occurred. These results again indicate that expression of MKP1 in H460 and H727 cells interferes with the activity of Bay11-7082 sensitization to cisplatin. Collectively, these results indicated that downmodulation of MKP1 expression in the assayed NSCLC cell lines was necessary to allow cisplatin sensitization by PI3K or NF-κB inhibition.

3.5. p65/relA and relB are expressed in NSCLC tumor specimens and Bortezomib inhibits lung cancer cell growth

To complement the studies described above regarding NF-κB role, we assayed by IHC the expression of nuclear NF-κB proteins in NSCLC tumor samples. Previous work has described the presence of p65 (canonical pathway) in surgically resected NSCLC specimens [32,33], but there are no reports on relB expression (non-canonical pathway) in this tumor. We evaluated the expression of p65/relA and relB in sections of tumor samples from 26 patients with NSCLC (Table 1 Supl 1). Nearly 60% of the samples corresponded to squamous cell carcinomas, 30% to adenocarcinomas, and the rest to undifferentiated large-cell carcinomas. Briefly, 60% of the samples were grouped into stage I, and rest into stage II. In tumor tissues, nuclear p65/relA staining was present in 50% of samples (Fig. 6A and B and Table 2). In contrast, relB nuclear staining was observed in 20% of samples. Samples that were positive for nuclear relB expression were also positive for nuclear p65/relA expression ($p = 0.0007$). Staining for p65/relA was found in the nuclei and at a lower level in the cytoplasm. In contrast, relB was barely detectable in the cytoplasm of the p65/relA-positive samples. These results agree with the western blots of nuclear extracts of H1299, H23 and A549 cells (Fig. 2A) and show that the expression of p65/relA and relB in the nucleus was homogeneously distributed among the different subgroups of patients. On the other hand, MKP1 expression was observed in 80% of the samples and was homogeneously distributed among different tumor subtypes (Table 2). Interestingly, 93% of tumors showing nuclear p65 or relB expression also expressed MKP1, indicating that at least 53% of the samples examined resemble the situation found in H727. A small percentage of the samples showed exclusively p65 and relB expression (like H1299 cells), and the rest of the samples were positive only for MKP1 (like H460 cells).

Based on these results, an in particular on the co-expression of NF-κB members representative of both the canonical and non-canonical pathways, we then tested the effect of Bortezomib in our panel of NSCLC cell lines. Bortezomib is a non-specific NFkB inhibitor that acts by means of proteasome inhibition, and is already in the clinic for the treatment of myeloma and lymphoma patients. We first tested, by using the HIV-luciferase reporter, the effect of Bortezomib treatment on NF-κB activity induced by TNF-α. As observed in Fig. 6C, Bortezomib was able to inhibit TNF-α-NF-κB, dependent activity in a time dependent manner in both H460 and H1299 cells. Similar results were obtained with the other three

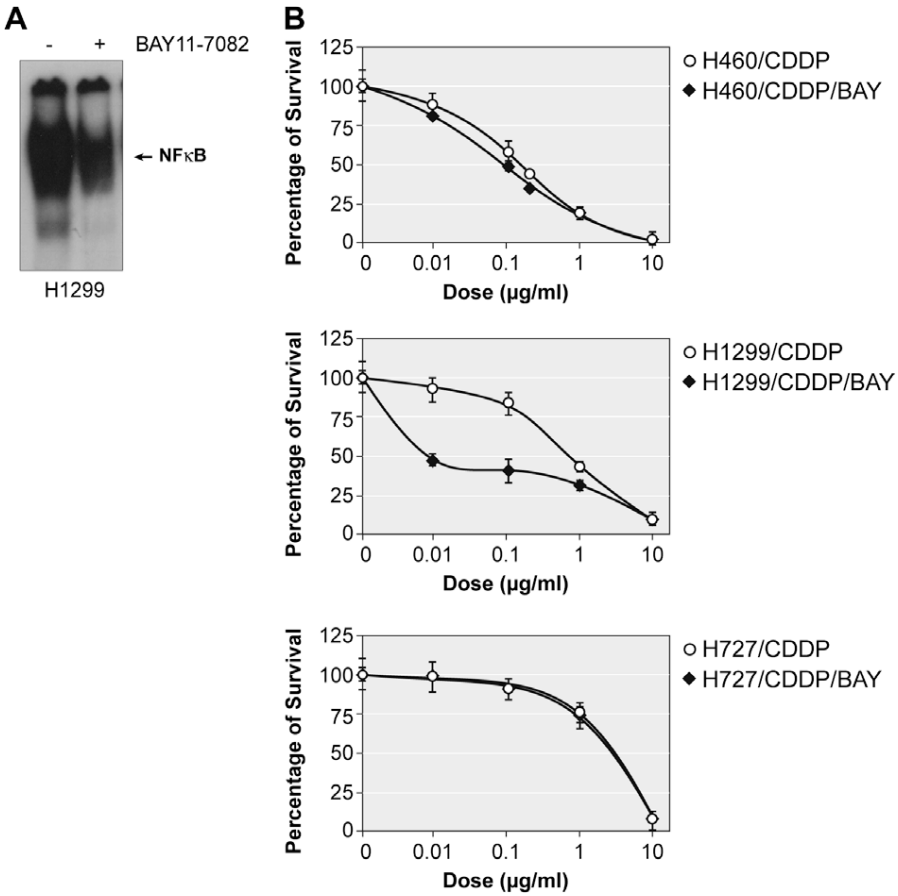

Fig. 4. Effect of the NFkB inhibitor BAY11-7082 treatment in NSCLC cells. (A) Effect of treatment with BAY11-7082 on nuclear NF-κB complexes in H1299 cells. H1299 cells were seeded and then incubated in serum-depleted medium for 16 h. Cells were pretreated with BAY11-7082 (5 μM) during 30 min. Nuclear extracts were obtained and analyzed by EMSA for NF-κB-binding activity as indicated in Materials and Methods. (B) Cell viability in response to cisplatin (at the indicated concentrations) of cell lines indicated in B and the effect of BAY11-7082 pretreatment. All cell lines were seeded in 24-well plates and cisplatin was added the following day at concentrations ranging from 0 to 10 μg/ml. When indicated (BAY), cells were pretreated for 4 h with BAY11-7082 (5 μM). After 72 h, the cells were fixed with glutaraldehyde, stained with crystal violet, and the percentage staining calculated according to standard procedures. Data represent the means of two experiments performed in quadruplicate.

cell lines (data not shown). We next treated all NSCLC cell lines with Bortezomib and found that it inhibited cell viability in most NSCLC cell lines used (Fig. 6D). Interestingly Bortezomib cytotoxic activity correlated with the basal NF-κB activity of the cells (Fig. 2D). H23 cells, with the highest basal NF-κB activity were the most sensitive, followed by H1299. A549 and H727 cells showed intermediate levels of sensitivity and also of NFkB activation. H460 cells that showed very low NF-κB activity were very resistant to Bortezomib. Bortezomib was indeed inhibiting proteasome activity, as shown in our results of proteasome proteolytic activity in NSCLC cells (data not shown). Furthermore we found that H460 cells, that were resistant to Bortezomib, when inhibiting MKP1 expression (H460cri), did not become sensitive to either Bortezomib or to the combination Bortezomib/cisplatin (data not shown) indicating that inhibition of proliferation even if was correlating with NF-κB activity on the cell lines used it was occurring by and MKP1 independent mechanism.

4. Discussion

Lung cancer is the leading cause of cancer death throughout the world and causes around 1.2 million deaths annually. The basis for this lethality is related to the late diagnosis (more than 70% of patient will be diagnose with an un-resectable disease) and the inherited resistance of lung cancer cells to therapeutic agents. However, data from phase III studies confirm that more than 60% of NSCLC patients will not respond to any approach and responders will always progress in a few months and die from this disease within a year.

The sensitivity of cells to chemotherapeutic drug-induced apoptosis appears to be dependent on the balance between pro-apoptotic and antiapoptotic signals. We have shown previously that overexpression of MKP1 in NSCLC occurs frequently in surgical samples from stage I/II patients [9]. NSCLC cell lines constitutively expressing MKP1 are more resistant to cisplatin than are cells in which MKP1 expression has been inhibited by siRNA transfection. Other signaling pathways altered in NSCLC may also influence the cisplatin response, such as inhibitors of drug-induced apoptosis. The PI3K/Akt pathway is a critical pathway in lung cancer [18] because it is constitutively activated in NSCLC and is detected in precursor lesions in

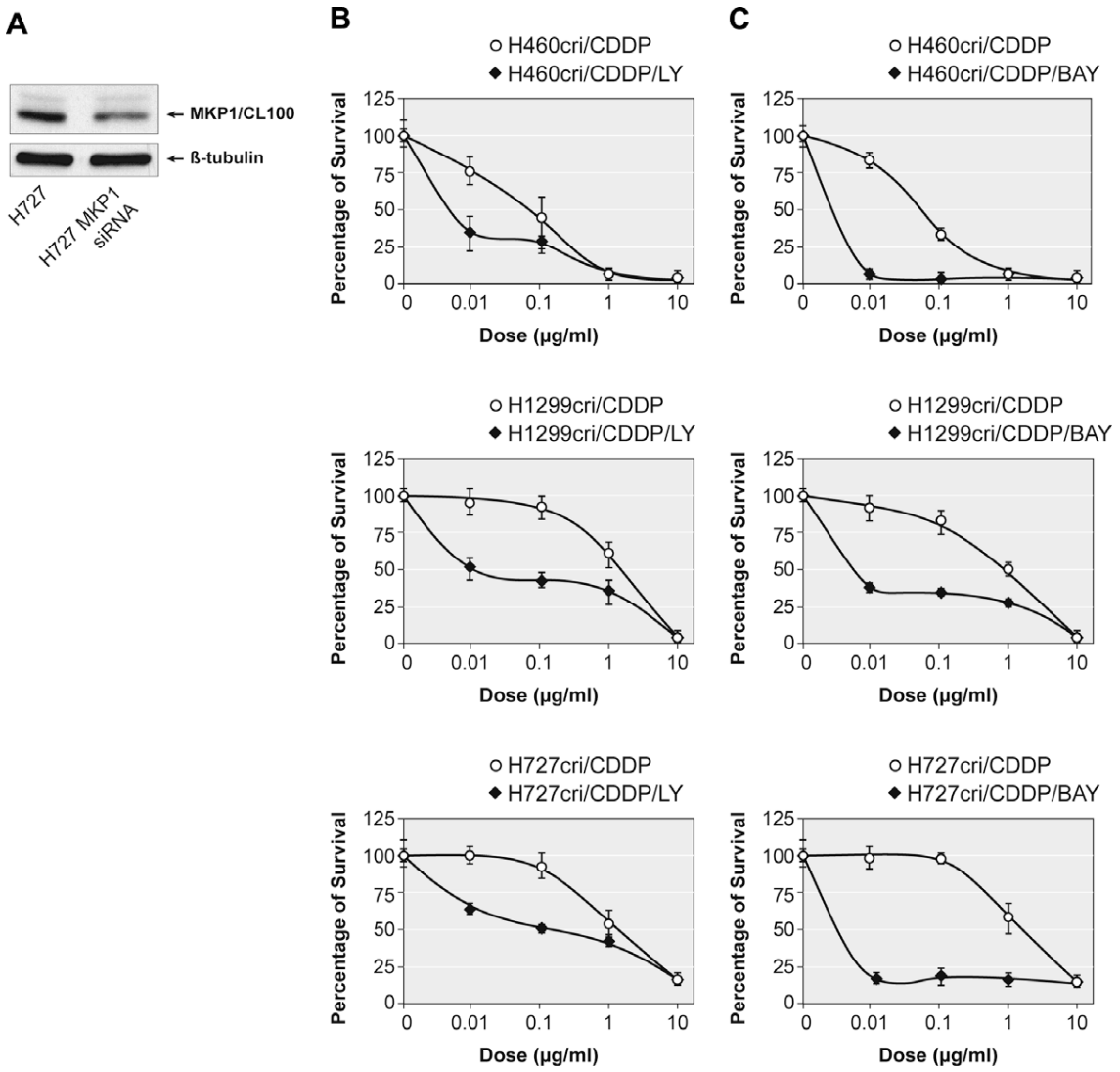

Fig. 5. Effect of pretreatment with the PI3K and NF-κB inhibitors on cisplatin survival in MKP1siRNA-derived NSCLC cells. (A) Levels of MKP1 protein in H727-MKP1-siRNA cells. Total protein (20 μg) of H727 cells transfected with either pSuperRetro or MKP1siRNA was separated by western blotting, and the resulting membrane was probed with specific antibody against MKP1. Antibody against β-tubulin was used to control for the amount of protein loaded. (B and C) Cell viability in response to cisplatin of H460 MKP1 siRNA (H460cri), H1299-MKP1-siRNA (H1299cri) and H727-MKP1-siRNA (H727cri) cells and effect of LY294002 (B) or BAY11-7082 (C) pretreatment. All cell lines were seeded in 24-well plates, and cisplatin was added the following day at a concentration of 0–10 μg/ml. When indicated (BAY or LY), cells were pretreated for 4 h with LY294002 (10 μM) or BAY11-7082 (4 μM). After 72 h, the cells were fixed with glutaraldehyde and stained with crystal violet, and the percentage staining calculated according to standard procedures. Data represent the means of two experiments performed in quadruplicate.

lung cancer [32,33]. In contrast, PI3K activity is also stimulated by carcinogens, such as components of tobacco, and in response to chemotherapy [34]. We have shown previously that treatment of cells with cisplatin induces cell death by modulating both survival and pro-apoptotic pathways (MKP1 and NF-κB) [27]. Activation of JNK induces AP1-mediated FasL transcription that contributes to cell death. In parallel, cisplatin also activates NF-κB, which activates transcription of genes such as *XIAP* and impairs cisplatin-induced apoptosis [27].

We investigated the role of the three pathways MKP1, Akt, and NF-κB in five different NSCLC cell lines to gain a better perspective of the potential variety of molecular contexts in tumors and found critical differences in the behavior of these cell lines after treatment with cisplatin. Although in all cell lines stimulation with cisplatin caused activation of JNK and p38, H1299 and H23 cells exhibited a faster kinetic, probably because these cells do not express (H1299) or expressed low levels (H23) of MKP1. In contrast, both H1299 and H727 cells, which are more resistant to cisplatin, expressed a basal level of phosphorylated Akt. Akt is an antiapoptotic protein, and its constitutive activation is consistent with the response to cisplatin in both cell lines. Nuclear expression of the NF-κB proteins, p65, and

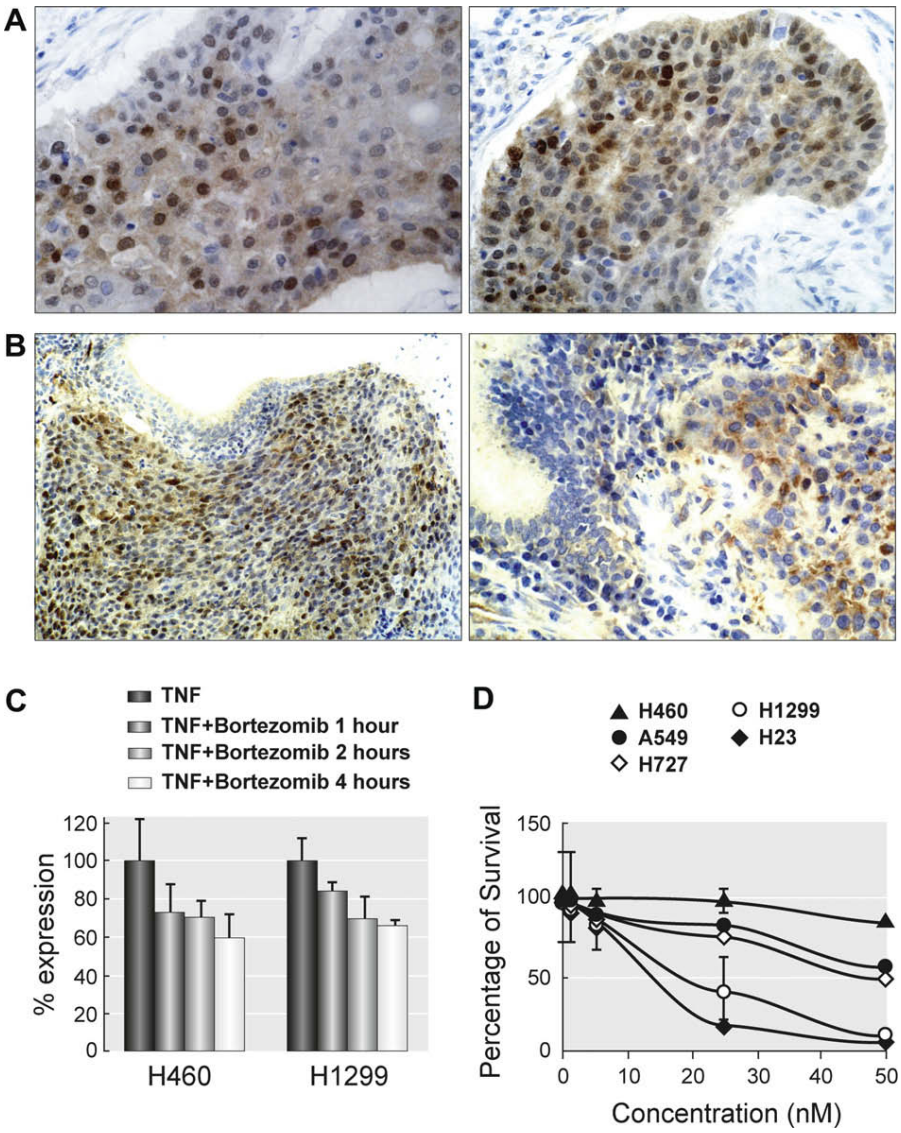

Fig. 6. Immunohistochemistry of patient tumor samples showing localization of relB and p65/relA and effect of Bortezomib treatment on NSCLC cells. (A and B) Sections of 5 μm thickness were cut from formalin-fixed, paraffin-embedded tissue blocks of the surgical tissues obtained from the patients. The primary antibody was incubated with the tissue slides and visualized with peroxidase-based EnVisionTM kit. The indicated samples were (A) hybridized with relB antibody and (B) hybridized with relA/p65. (C) H460 and H1299 cells were seeded en 60 mM dishes and transfected with 0.5 μg of (−453/−80) HIV-luc. Cells were pretreated or not with Bortezomib and then with TNF-α for the indicated times. The fold increase in luciferase activity was calculated relative to the value for the cells non-treated with Bortezomib. (D) Cell viability of NSCLC cells with Bortezomib. H460, H1299, H727, A549 and H23 cells were seeded in 24-well plates and Bortezomib was added the following day at concentrations ranging from 0 to 50 nM. After 72 h, the cells were fixed with glutaraldehyde, stained with crystal violet, and the percentage staining calculated according to standard procedures. Data represent the means of two experiments performed in quadruplicate.

relB is increased in A549, H1299, H727 and H23 and low in H460 cells. The increased expression correlated with an increase in NF-κB-dependent transcription on an HIV-reporter vector in H1299, H23, H727 and A549 cells. Interestingly, this increase in the KB dependent transcription of these proteins seems to occur in H727 and A549 cells because of transcriptional activation of p65. Although increased nuclear levels of relB were detected in H1299, H23 and A549 cells, we could not detected relB bound to kB complexes in gel retardation assays.

A relevant finding of this work was that more than one survival-signaling pathway is involved in regulating the altered response to cisplatin in NSCLC cells. We have previously described that inhibiting MKP1 expression in H460 sensitizes cells to cisplatin [9]. Treatment with cisplatin also induces activation of Akt, whose inhibition results in increased cytotoxicity, but only when MKP1 expression is inhibited. Because cisplatin also induces activation of NF-κB [27], similar dependence on MKP1 expression was observed in cells treated with the NF-κB inhibitor BAY11-

7082. This effect also occurred in other cell lines such as H727 cells, which expressed basal levels of MKP1. In contrast, when MKP1 was not expressed at basal levels, independent inhibition of either Akt phosphorylation or NF-κB activity sensitized H1299 cells to cisplatin. Taken together, these results indicate that in the presence of basal levels of MKP1, this protein predominates in the regulation of the response to cisplatin responses and that this effect appears to be independent of PI3K or NF-κB pathways. These findings suggest that MKP1 is a good target for improving the cellular response to chemotherapy. Since NF-κB represent a survival pathway in NSCLC cells, we used another strategy to inhibit NF-κB activity by inhibiting the proteasome activity with Bortezomib. Interestingly treatment of NSCLC with Bortezomib was cytotoxic only in those cells that harbor high NF-κB-constitutive activation.

Analysis of surgical samples from NSCLC patients indicated that in most patients (80%) MKP1 levels are upregulated [9] and that only in a small percentage of patients (1/26) only NF-κB (p65/relA and relB) localizes in the nuclei. p65/relA has been implicated in both tumor progression and cell resistance to chemotherapy. Inhibition of NF-κB increases the efficacy of cisplatin in ovarian cancer models [35] and cervical cancer [36], to 5-fluorouracil in colon cancer [37], and to adjuvant therapy in breast cancer [38]. On the other hand, some compounds, such as 17AAG, synergistically potentiate TNF-α-induced lung cancer cell death by blocking NF-κB [39]. These findings support the potential improvement in cisplatin responses by co-targeting NF-κB or Akt and MKP1. Our results indicate that at least 25% from the surgical samples of NSCLC expressed nuclear relB or p65, an indicative of constitutive NF-κB activity and therefore would be good candidates to benefit from Bortezomib treatment. So, it would be of interest to evaluate cisplatin activity in the adjuvant setting in patients selected by our molecular approach in order not to expose patients with scarce molecular possibilities to respond to cisplatin to an almost certain toxicity. Finally, clinical data from the unique reported phase II study where Bortezomib was administered to NSCLC patients both alone and in combination with docetaxel, evidenced a modest activity as single-agent and did not enhance docetaxel responses or time- to- progression [40]. In this regard, our molecular data support a new evaluation of Bortezomib activity in NSCLC patients selected by our molecular approach, since other studies tested this drug in molecularly inadequate patients.

In conclusion, our results showed that an activated PI3K/Akt or NF-κB pathway was linked to a reduced sensitivity to cisplatin in NSCLC cells. Inhibition of PI3K or NF-κB potently enhanced cisplatin cytotoxicity in cells with endogenous or genetically induced low MKP1 levels. We believe that these data add support to the concept of MKP1 as a novel target for NSCLC treatment and that assessment of MKP1 expression emerges as a biomarker for chemosensitizing strategies towards cisplatin in NSCLC.

Conflicts of interest

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2009.05.029.

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Material suplementario

Immunohistochemistry

Formalin-fixed and paraffin-embedded blocks from 32 surgical biopsies of NSCLC were selected for MKP1, relB, and p65/relA immunohistochemistry. The blocks included, whenever possible, invasive carcinoma and nontumorous surrounding epithelium. Institutional approval was received for the conduct of the study. Sections of 5 μm were cut from formalin-fixed, paraffin-embedded tissue blocks from the patients. Slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% H2O2 in methanol for 10 min at room temperature (RT). Antigens were retrieved by incubation in EDTA for 45 min at 155 °C. The primary polyclonal antibodies to MKP1, p65, or relB were diluted at 1:200 in 1% bovine serum albumin in TBS. Tissue slides were incubated with the primary antibodies for 1 h at RT. Slides were rinsed in TBS and incubated with the peroxidase-based EnVision™ kit (Dako Corp., Carpinteria, CA) for 30 min at RT. Specimens were next incubated with diaminobenzidine chromogenic substrate (Dako Corp.) for 5 min at RT. Sections were counterstained with hematoxylin, stepwise dehydrated through graded alcohols, and cleared in xylene. Immunostaining was evaluated by scoring by an expert surgical pathologist. For MKP1 staining we have used as positive and negative controls MEFs derived from MKP1 KO mice and the respective wild type controls. In these experiments with the wt MEFs we observed a positive nuclear staining which was absent in the KO MEFs. The pattern of immunostaining in the samples was considered a qualitative variable and recorded as negative (absence of nuclear expression) or positive (presence of nuclear expression) for each protein (RelA, RelB, and MKP1). Correlations were evaluated with the chi-square test and classical corrections were made when necessary. All statistics analysis was performed on SPSS 13.0 software.

Table 1. Baseline characteristics of patients and tumors included.

CHARACTERISTIC		N
Patients		26
Sex	Male	24
	Female	2
Age, mean years (range)		62 (42–79)
Histological subtype	Adenocarcinoma	8
	Squamous	15
	Undifferentiated large cell carcinoma	3
Stage	I	16
	II	10

Table 2. Distribution of p65/relA and relB in tumor tissues according to stage and histology. relA expression was significantly correlated with relB expression (P = 0.007), showing that the presence of relA is required for relB immunostaining. No other correlations were observed.

					STAGE			
					I		II	
Histology	n	relA	relB	relA+relB	relA/ MKP1	relB/ MKP1	RelA/ MKP1	relB/ MKP1
Squamous-cell carcinoma	15	8 (53%)	4 (26%)	4 (26%)	6/6	3/2	3/3	1/1
Adenocarcinoma	8	3 (37%)	0	0	2/2	0/0	1/1	0/0
Large-cell carcinoma	3	1 (33%)	1 (33%)	1 (33%)	0	0	1/1	1/1
n	26	12 (46%)	5 (19%)	5 (19%)	8/8	3/2	5/5	2/2

DISCUSIÓN

El cáncer de pulmón es uno de los tipos de cáncer que más muertes causan al año en el mundo. Las causas de esta mortalidad están relacionadas con una diagnosis tardía (más de un 70% de los pacientes se diagnostica cuando la enfermedad es inoperable) y con la resistencia de las células tumorales a los agentes terapéuticos. Actualmente la terapia que se utiliza para el tratamiento de pacientes con CNMP con buen pronóstico está basada en una combinación de fármacos que incluye cisplatino, sin embargo, más de un 60% de pacientes con esta enfermedad no responderá a ningún abordaje según datos de un estudio en fase III (Schiller et al., 2002). El CDDP tiene una acción antitumoral amplia, mientras que su toxicidad en tejidos normales está relativamente restringida a tipos celulares específicos, como a las células enterocromafines gastrointestinales, las células de los túbulos renales, y las células de los ganglios espinales (McKeage, 1995). Sin embargo, la exposición a CDDP está asociada a la inducción de la hipermetilación del ADN aunque se desconocen los mecanismos por los que esto sucede. El CDDP no tiene efectos directos sobre la metilación del ADN, ya que es un agente intercalante que funciona uniéndose al ADN mediante enlaces covalentes intra e inter catenarios; sin embargo, cada vez es mayor la evidencia de que hay un nexo entre la pérdida de expresión de genes en respuesta al tratamiento con CDDP por metilación del ADN y la resistencia al fármaco. De hecho, la exposición a CDDP de células tumorales de adenocarcinoma de pulmón (HTB-54), de rabdomiosarcoma (CCL-136) y de ovario (A2780) induce la hipermetilación del ADN (Nyce, 1989, Nyce, 1997, Zeller et al., 2012), un evento que también se observa *in vivo* (Koul et al., 2004, Chang et al., 2010). Entre las posibles causas por las que el CDDP puede producir metilación del ADN, estaría la producción de especies reactivas de oxígeno, que se originan como respuesta a la toxicidad del metal que provoca el CDDP (Ferroni et al., 2011, Attia, 2012, Khan et al., 2012). El estrés oxidativo induce una compleja respuesta celular que incluye alteraciones genéticas y epigenéticas, que pueden producirse tanto a nivel de un aumento en la expresión de las DNMTs (Campos et al., 2007), como por cambios en la acetilación o metilación de histonas (Lawless et al., 2010). Sin embargo, se desconoce el mecanismo exacto por el que se produce la metilación del ADN en respuesta a CDDP.

Identificación de genes implicados en la resistencia a CDDP, validación de su expresión y regulación epigenética

Muchos de los estudios sobre la relación entre la resistencia adquirida a quimioterapia y las alteraciones epigenéticas, se han centrado en un número limitado de genes candidatos, como *CDKN2A* (Katsaros et al., 2004); *RASSF1A* (Makarla et al., 2005), *hMLH1* (Strathdee et al., 1999) los cuales contribuyen al proceso de resistencia en cáncer de ovario, de pulmón y otros. En este trabajo se ha planteado un abordaje global, desarrollando una estrategia basada en la tecnología de microarrays para identificar nuevas dianas potenciales de la hipermetilación de promotores en los fenotipos resistentes a CDDP en cáncer de pulmón. Se establecieron las líneas celulares H23R y H460R, a partir de sus respectivas líneas parentales sensibles al fármaco, con un índice de resistencia a CDDP cercano al que presentaba la línea 41R (Mellish and Kelland, 1994, Kelland et al., 1992) asumiendo que así se producirían unos eventos de resistencia similares en las tres líneas celulares. Las líneas resistentes de CNMP fueron sometidas a un tratamiento de reactivación epigenética, y comparadas con las líneas parentales y resistentes sin tratar mediante análisis de microarrays. A través de la combinación de los análisis estadísticos

y ontológicos, se identificó un grupo de genes implicados en rutas de progresión tumoral, similar a una aproximación genómica publicada recientemente, que identifica la apoptosis como la ruta más representada en resistencia a CDDP (Riedel et al., 2008). Se validaron nueve genes seleccionados de este grupo, éstos tenían unos niveles de expresión menor en las líneas resistentes que en las líneas parentales y se re-expresaban tras el tratamiento epigenético; de ellos, tres eran comunes en las dos líneas de CNMP *(ARRDC-4, DKK1* y *DUSP5),* mientras que los otros 6 genes eran específicos de cada línea, tres para H23 *(AREG, GDF15* y *IGFBP-3)* y tres para H460 *(NNAT, PHLDA1* y *S100A2),* indicando que el CDDP afectaba a diferentes grupos de genes y/o diferentes rutas biológicas dependiendo de la línea tumoral, lo cual podría explicar la gran variación inter-individual en la respuesta a drogas que existe en pacientes de cáncer de pulmón. Con la validación se confirmó que todos los genes se expresaban en condiciones basales en las células parentales, lo que indica su importancia en el mantenimiento del fenotipo sensible, además, su expresión se perdía o disminuía en resistencia. Es interesante resaltar que la expresión disminuida de uno de estos nueve genes, el gen *DKK1,* ha sido asociada recientemente con la resistencia adquirida a CDDP en cáncer de cabeza y cuello (Gosepath et al., 2008). Por otra parte, la validación epigenética reveló que un gen de nuestro panel, *IGFBP-3,* tenía metilado el promotor sólo en los tipos celulares resistentes; indicando que el resto de genes no estaban bajo regulación epigenética directa. Sin embargo, éstos se reexpresan después del tratamiento con 5-AZAdC y TSA lo que indica que estos genes podrían estar regulados por una proteína o por un factor de transcripción cuya expresión fuese reactivada por el proceso de desmetilación del ADN. Esto es lo que ocurre con el gen *TGM2,* que aunque se expresa en células tumorales de riñón tras el tratamiento epigenético, no se debe a que esté metilado su promotor sino a la regulación que sufre por parte de RASSF1, el cual si que está regulado epigenéticamente (Ibanez de Caceres et al., 2006).

La metilación del promotor de *IGFBP-3* media la respuesta a CDDP

Nuestros resultados mostraron que *IGFBP-3* está silenciado por la hipermetilación de su promotor en las líneas tumorales resistentes 41R y H23R en comparación con las respectivas células parentales, que tienen una marcada expresión basal de *IGFBP-3*. Además, el tratamiento con agentes desmetilantes e inhibidores de desacetilasas de histonas, restauró tanto las posiciones CpG no metiladas, como la expresión del gen, confirmando la regulación epigenética de *IGFBP-3* en las líneas resistentes a CDDP. Estos resultados confirmaban la correlación entre la expresión de *IGFBP-3* regulada por la hipermetilación del promotor y la pérdida de sensibilidad a CDDP. Además, se demostró que la metilación del promotor de *IGFBP-3* es la causa y no la consecuencia de la adquisición de resistencia a CDDP. Para estudiar esto, se inhibió la expresión de *IGFBP-3* en las células 41S, lo que produjo una fuerte bajada en la sensibilidad a CDDP, con un índice de resistencia intermedio, que se encontraba entre el de las células sensibles (41S) y el de las células resistentes (41R) transfectadas con el ARN de interferencia control. Por el contrario, la reactivación epigenética de la expresión de *IGFBP-3* incrementaba la sensibilidad a CDDP en las células 41R, apoyando nuestra hipótesis.

Se estudió después la relación entre la sensibilidad/resistencia a CDDP y el estado de metilación del promotor de *IGFBP-3* en 23 líneas tumorales adicionales, observándose que de aquellas

líneas que presentaban una menor sensibilidad a CDDP, una gran mayoría (75%) tenían metilado el promotor de *IGFBP-3.* De las 23 líneas celulares, 16 mostraban resistencia a CDDP en distinto grado, quizá como consecuencia de sus orígenes metastásicos. Aún así, estos datos afianzan la relación entre la resistencia a CDDP y la metilación del promotor de *IGFBP-3* en diferentes tipos de tumores. Asimismo la sobreexpresión de *IGFBP-3* en cuatro de las líneas resistentes (HT29, H727, H1299 y PANC1) que además presentaban el promotor de *IGFBP-3* metilado, inducía un aumento en la mortalidad en estas células si las comparábamos con las transfectadas con el vector vacío.

A partir de estos resultados podemos confirmar que la hipermetilación del promotor de *IGFBP-3* podría tener un papel muy importante en la respuesta a CDDP. La inducción de la expresión de *IGFBP-3* por p53 está asociada a un aumento de la secreción de una forma activa de *IGFBP-3* capaz de inhibir la señalización mitótica de IGF-I (Buckbinder et al., 1995). El análisis citogenético de las líneas celulares 41S y 41R no indicaba cambios en el cromosoma 17 donde se encuentra localizado el gen de p53 (17p13) (Leyland-Jones et al., 1999), indicando que la metilación del promotor de *IGFBP-3* en la zona de unión de p53 podría estar inhibiendo la posible inducción de la expresión de *IGFBP-3* por p53 en estas líneas celulares. La vía de señalización de IGF-I podría mediar resistencia a quimioterapia modulando la actividad de proteínas de supervivencia por la vía de señalización de PI3K/AKT, esto protegería a las células de CNMP de la apoptosis inducida por algunas drogas, como se ha reportado previamente con los inhibidores de la actividad tirosina quinasa de EGFR, erlotinib y gefitinib (Morgillo et al., 2007, Morgillo et al., 2006). Estos autores sugieren que la resistencia a estos inhibidores tirosina quinasa (TKIs) está causada por la activación de mecanismos de supervivencia alternativos en la célula, ya que estas drogas no inhiben la proliferación al suprimir la activación de EGFR. La expresión disminuída de *IGFBP-3* en líneas resistentes, podría provocar un aumento en los niveles de IGF-I libre capaz de unirse a su receptor y activar así la señalización a través de IGF-IR, induciendo la supervivencia y manteniendo la proliferación en las líneas resistentes a CDDP.

La respuesta a CDDP está mediada por la expresión de *IGFBP-3* a través de la vía de señalización de IGF-IR/AKT

Para determinar si la resistencia a CDDP estaba mediada por la activación de la ruta PI3K/AKT, se utilizaron las tres líneas celulares pareadas sensibles/resistentes a CDDP, que como se ha visto previamente, presentaban diferentes niveles de expresión del gen *IGFBP-3* así como distinto grado de metilación de su promotor, además la resistencia a CDDP en las células 41R y H23R coincidía con la metilación del promotor de *IGFBP-3* y una expresión disminuida del gen. Sin embargo la línea celular H460R tenía el promotor de *IGFBP-3* no metilado, indicando que la resistencia a CDDP en estas células es independiente de la regulación epigenética de *IGFBP-3,* sirviéndonos como control negativo para el estudio. Confirmando nuestra hipótesis, la hipermetilación del promotor de *IGFBP-3* correlacionó con una fuerte activación de AKT en las células 41R. Las células H23S tenían el promotor de *IGFBP-3* semimetilado y mostraban una activación basal de AKT, dicha activación se ampliaba en el rango de dosis-respuesta a CDDP, cuando las células adquirían una mayor densidad de metilación del promotor de *IGFBP-3* como resultado del proceso de establecimiento de la línea celular H23R. Como esperábamos no

se observaron cambios en la línea celular H460. Se descartó el posible papel de PTEN en el proceso de resistencia a CDDP en estas líneas, ya que no encontramos alteraciones de sus niveles de expresión al comparar las células H460S/R y 41S/R. La línea celular H23 que tiene una mutación sin sentido en *PTEN* (PTEN R233) (Forgacs et al., 1998), mostraba unos niveles casi indetectables de la proteína. De hecho, aunque la expresión de *PTEN* puede ser alterada por metilación de su promotor, mutaciones y deleciones en tumores humanos, esto ocurre con muy baja frecuencia en CNMP (Forgacs et al., 1998, Yokomizo et al., 1998). Estos resultados indican que la hipermetilación del promotor de *IGFBP-3* induce la fosforilación de AKT en líneas celulares tumorales humanas. De hecho, la actividad de AKT es la responsable en parte, de la resistencia a CDDP en estas células, ya que la sobreexpresión de *IGFBP-3* disminuía los niveles de fosforilación de AKT, induciendo un incremento en la sensibilidad a CDDP en las células 41R y un índice de resistencia intermedio entre el de las células 41S y el de las células 41R. También se observó el papel directo de la actividad de AKT en la resistencia adquirida a CDDP, ya que tanto la inhibición de la fosforilación de AKT como la inhibición de su expresión inducían un incremento en la mortalidad celular y en la sensibilidad a CDDP en las células 41R. Estos resultados indicaban que la fosforilación de AKT tiene un papel importante en el proceso de supervivencia frente a CDDP en las células 41R, que concuerda con los datos de la activación de la vía de AKT en resistencia a CDDP en células de cáncer de pulmón (Hamano et al., 2011). Con lo que estos resultados nos revelan que la metilación del promotor de *IGFBP-3* es un evento que media la resistencia a CDDP a través de la activación de AKT. El siguiente paso fue observar qué receptor activaba la vía de AKT en el proceso de resistencia adquirida a CDDP. Encontramos niveles bajos del receptor de EGF en las células 41R en comparación con las células parentales sensibles 41S, este efecto de la disminución de los niveles del receptor de EGFR en respuesta a CDDP también se ha observado el las células MDA-MB-468 (Oliveras-Ferraros et al., 2008). Contrariamente, IGF-IR se encontraba activado sólo en las células 41R, probablemente porque en las células 41S, en las que *IGFBP-3* se expresa con normalidad, el factor de crecimiento IGF-I se encuentra secuestrado y por tanto estaría bloqueada la activación de su receptor. De hecho, IGF-I media una fosforilación sostenida de AKT, lo cual es esencial para la supervivencia a largo plazo en la protección contra los agentes tóxicos en la células de la glía (Romanelli et al., 2007).

Además, la activación de IGF-IR era menor en las células 41R cuando se aumentaban los niveles de expresión de *IGFBP-3,* confirmando el papel de *IGFBP-3* en la activación de AKT a través de la fosforilación del receptor IGF-IR. Finalmente se corroboró por inmunohistoquímica la presencia en la membrana citoplasmática de IGF-IR, y se observó una internalización parcial del receptor después de estimular las células 41S con IGF-I, mientras que en las células 41R, en las que IGF-IR en condiciones basales tenía una localización intracelular, el tratamiento con el ligando IGF-I la incrementaba drásticamente, debido a una internalización del receptor (Vecchione et al., 2003). La activación crónica del receptor IGF-IR en la línea celular 41R podría deberse a la disminución de los niveles de IGFBP-3, que causaría una exposición continua de las células a IGF-I. El estímulo con este factor de crecimiento podría finalmente provocar la activación sostenida de AKT observada en las líneas a través de la internalización de IGF-IR y de su reciclaje (Romanelli et al., 2007). Por otra parte, aunque no encontramos ningún cambio en los niveles de ARN mensajero de EGFR entre las líneas 41S y 41R, la señalización de este receptor en las células 41R es muy tenue con respecto a las células 41S, indicando que las alteraciones podrían ocurrir

a un nivel postraduccional, confirmado así los datos obtenidos previamente por la técnica de Western Blot. Estos resultados explicarían por qué en ensayos clínicos en fase III, cuando se combina la terapia estándar con gefitinib (inhibidor de EGFR), ésta falla en pacientes de CNMP en estadios avanzados (Takeda et al., 2010).

Estos resultados no eran específicos de las líneas 41S/41R, ya que la sobreexpresión de *IGFBP-3* en líneas tumorales de pulmón y páncreas H1299 y PANC-1, que en condiciones basales tienen silenciada la expresión del gen por hipermetilación de su promotor, inducían un notable descenso en la fosforilación de IGF-IR después del tratamiento con CDDP, que concordaba con una bajada en los niveles de pAKT. El mismo perfil con respecto a pAKT se observaba en la línea H727. Estos resultados mostraban que la regulación de la vía de IGF-IR por la expresión de *IGFBP-3* es probablemente un mecanismo común en la sensibilidad a CDDP en diferentes tipos de tumores. Además la sobreexpresión de *IGFBP-3* también inducía un descenso de la supervivencia en las líneas tumorales humanas ensayadas, que tenían el promotor de *IGFBP-3* metilado, ya que se observó que la sobrexpresión de *IGFBP-3* provocaba una mayor mortalidad en aquellas células que presentaban una mayor resistencia a CDDP. Estos resultados confirman una fuerte correlación entre la expresión de *IGFBP-3* y la mortalidad celular en respuesta a CDDP. Explicado en términos de una posible aplicación traslacional, aquellos tumores que pierden la expresión de *IGFBP-3* podrían ser dianas de una terapia con agentes desmetilantes, debido a la conexión entre la resistencia a CDDP y la mortalidad celular esperada después de la expresión de *IGFBP-3.*

La metilación del promotor de *IGFBP-3* es específica de tumores primarios de CNMP resistentes a CDDP y su combinación con el estado de activación de IGF-IR, EGFR y/o AKT predice resistencia a CDDP en muestras de CNMP

Finalmente quisimos trasladar los resultados obtenidos en el laboratorio a la clínica, así a partir de un panel de 36 pacientes en cuyas muestras se valoró el estado de metilación del promotor de *IGFBP-3*, observamos en primer lugar que la metilación aberrante del promotor de *IGFBP-3* podía predecir la sensibilidad a CDDP en tumores primarios. Para ello se midió la viabilidad de las muestras a CDDP in vitro y se determinó cuales eran resistentes o sensibles, y se encontró que muchos de los dinucleótidos CpG se encontraban metilados en muestras resistentes pero no en las sensibles, indicando una correlación significativa entre la metilación de *IGFBP-3* y la respuesta al quimioterápico CDDP. Además, si se seguía la historia clínica de estos pacientes después del tratamiento con quimioterapia, aquellos diagnosticados con un estadio I y que no tenían metilado el promotor de *IGFBP-3,* presentaban una clara mayor supervivencia libre de enfermedad, comparado con otros grupos. El tamaño de la muestra no fue lo suficientemente grande como para demostrar de forma significativa la diferencia entre los grupos, pero si para tener un punto de partida para futuros estudios. Sin embargo, en los datos de los pacientes en estadio II encontramos un escenario opuesto al anterior, que puede ser debido también al tamaño muestral, aunque es posible que una terapia adyuvante con la que normalmente se trata a pacientes en estadio II pueda tener un papel en las diferencias observadas. Desafortunadamente, no se obtuvo información sobre la terapia adyuvante utilizada en estos pacientes.

Finalmente quisimos estudiar qué rutas celulares estaban implicadas en la resistencia a CDDP en estas muestras. Así encontramos una activación predominante de IGF-IR pero no de EGFR en estos pacientes de CNMP, siendo el porcentaje de muestras que presentan pEGFR similar al publicado anteriormente por otros autores (Sonnweber et al., 2006). Además se observó que la activación del receptor de IGFI fue un evento frecuente en tumores primarios de CNMP que eran resistentes a CDDP, y que en aquellos pacientes con IGF-IR fosforilado se mantenía AKT fosforilada. Estos resultados podrían explicar los datos previos que mostraban la activación constitutiva de AKT a través de la activación de IGF-IR, pero no de EGFR en células resistentes a la inhibición de EGFR (Judde et al., 2007). Asimismo, la metilación de *IGFBP-3* y la fosforilación de IGF-IR y AKT ocurrían sólo en pacientes de CNMP resistentes a CDDP, indicando que la deficiencia en la expresión *IGFBP-3* derivada de la metilación de su promotor podría mediar resistencia a CDDP en pacientes de CNMP a través de la activación de la vía de señalización IGF-IR/AKT. Por otra parte, la combinación de la metilación del promotor de *IGFBP-3* con el estado de activación de IGF-IR, y/o AKT, y/o EGFR podría usarse para predecir resistencia o sensibilidad a CDDP en muestras de CNMP. De hecho, en este trabajo presentamos un test predictivo que considera que una muestra necesita al menos dos modificaciones de estas cuatro: la metilación del gen *IGFBP-3,* la fosforilación de IGF-IR, la fosforilación de EGFR y la fosforilación de AKT, para obtener un verdadero cambio en el fenotipo en términos de respuesta a CDDP con una exactitud y una especificidad de 0,84 y 0,9 respectivamente, y con un ratio de probabilidad positiva de 9,75.

Estudio del estado de las vías de señalización de PI3K/AKT y NFκB y de la expresión de MKP1 en líneas tumorales de CNMP

A parte de estos resultados sobre la metilación del promotor de *IGFBP-3* y su relación con la adquisición de resistencia a CDDP, en nuestro grupo se había demostrado también una relación entre la expresión de la fosfatasa MKP1 y resistencia a dicho fármaco. Observamos que la sobreexpresión de MKP1 en CNMP ocurre frecuentemente en muestras quirúrgicas de pacientes en estadio I/II y también que las líneas celulares de CNMP que expresan de forma constitutiva MKP1 son más resistentes a CDDP, que la misma línea celular con la expresión de MKP1 inhibida (Chattopadhyay et al., 2006). Existen otras rutas celulares alteradas en CNMP que también parecen afectar la respuesta a CDDP, como la ya comentada ruta PI3K/AKT, que puede encontrarse constitutivamente activa en CNMP debido a mutaciones, amplificaciones o deleciones de diferentes componentes de la vía, o como hemos observado, a cambios en la expresión de *IGFBP-3* por la metilación de su promotor. Igualmente, el tratamiento de las células con CDDP promueve muerte celular por modulación de rutas de supervivencia y proapoptóticas; por una parte la inducción de JNK y la transcripción del ligando Fas mediada por AP-1 provoca muerte celular, y por otra, el CDDP activa NFκB, el cual activa genes como *XIAP,* que promueven supervivencia (Sanchez-Perez et al., 2002).

En esta parte del trabajo se estudió el papel de tres rutas: JNK/MKP1, PI3K/AKT y NFκB en cinco líneas celulares de CNMP, para así poder obtener una mejor perspectiva de la situación real en este tipo de tumores. Encontramos importantes diferencias en el comportamiento bioquímico de estas células al tratarlas con CDDP. Aunque en las cinco líneas celulares el CDDP inducía la activación de JNK y p38, en células H1299 y H23 apreciamos una activación temprana de dichas

quinasas, probablemente debido a que estas células expresan niveles muy bajos de MKP1. Por otra parte las células H1299 y H727, que eran las líneas más resistentes a CDDP, presentaba niveles altos de fosforilación de AKT en condiciones basales.

Estudiamos también la expresión de proteínas de la familia NFκB (p65/RelA y RelB) en el núcleo en condiciones basales, y encontramos niveles altos en las células H1299, A549 y H23, menores en las H727 e inexistentes en las células H460. Después observamos que la actividad transcripcional de NFκB era alta en las líneas celulares H1299, H727 y H23, según los datos obtenidos de la medida de la actividad luciferasa, siendo las líneas celulares H23 y H1299 las que presentaban una mayor actividad de NFκB en condiciones basales. También advertimos que la actividad luciferasa obtenida en las células H727 se debía principalmente a la activación del dominio de transactivación de p65/RelA, que puede deberse a que esta línea celular tiene altos niveles de AKT en su forma fosforilada (Ghobrial et al., 2005). La activación transcripcional inducida por RelB parece ser muy importante en las células A549 y H1299, en estas últimas este dato encaja con el obtenido en los ensayos de retardo de la movilidad electroforética, mientras que en las células A549, que tenían una débil activación transcripcional mediada por NFκB, ésta activación parecía corresponder principalmente a la vía no canónica.

Finalmente confirmamos que la inhibición de la expresión de MKP1 en la línea celular H460 sensibiliza las células a CDDP. El tratamiento con CDDP inducía la activación de AKT y NFκB y la inhibición de ambas vías aumentaba la citotoxicidad del fármaco pero sólo cuando la expresión MKP1 estaba inhibida. Estos resultados indican que la expresión basal de MKP1 en las células estudiadas modula la respuesta a CDDP, por lo que MKP1 podría ser una buena diana terapéutica en la respuesta celular a la quimioterapia basada en platinos.

Los factores de transcripción NFκB, p65/RelA y RelB se encuentran frecuentemente localizados en el núcleo de muestras de pacientes de CNMP. El inhibidor del proteasoma, bortezomib, inhibe el crecimiento de células de CNMP.

El análisis en muestras de pacientes de CNMP indicó que muchos de los pacientes (80%) tenían niveles altos de MKP1 (Chattopadhyay et al., 2006) y que sólo un pequeño porcentaje de los pacientes (1/26) presentaban exclusivamente las subunidades de NFκB, p65/RelA y RelB en el núcleo. P65/RelA están involucrados tanto en progresión tumoral como en resistencia a quimioterapia. La inhibición de NFκB incrementa la eficacia del CDDP en modelos de cáncer de ovario (Mabuchi et al., 2004) y cáncer cervical (Dejardin, 2006), a 5-fluoracilo en cáncer de colon (Voboril et al., 2004) y a terapia adyuvante en cáncer de mama (Montagut et al., 2006). Nuestros resultados también revelan que al menos un 25% de las muestras de pacientes con CNMP presentaban RelB o p65/RelA en el núcleo (además de MKP1, que localizaba en el núcleo con dichas proteínas en todas las muestras menos en una), lo que indica tanto una activación constitutiva de NFκB como que estos pacientes podrían ser unos buenos candidatos a beneficiarse de un tratamiento con bortezomib. Sin embargo, los datos clínicos obtenidos del único estudio en fase II donde el bortezomib era administrado en pacientes de CNMP, ya fuera sólo o en combinación con docetaxel, evidenciaban una actividad modesta como agente único y no aumentaba la respuesta que producía el docetaxel (Fanucchi et al., 2006).

La vía de señalización NFκB está implicada en supervivencia en células de CNMP, por lo que utilizamos otra estrategia para inhibir esta ruta, a través del uso de bortezomib. Ambas vías de activación de NFκB, la canónica y la no canónica, necesitan de la actividad del proteasoma para su señalización (Adams, 2004). En primer lugar, utilizamos bortezomib y TNFα, y mediante la medida de actividad luciferasa con el promotor reportero HIVluc que contiene tres sitios de unión para factores NFκB, confirmamos que el fármaco bortezomib inhibía la actividad transcricional de NFκB en células de CNMP. Además, cuando tratábamos las células de CNMP con bortezomib, la citotoxicidad de éste era mayor en aquellas líneas en las que habíamos observado niveles transcripcionalmente altos de NFκB.

Estos últimos resultados indican que tal vez aquellos pacientes que presenten p65/RelA o RelB localizados en el núcleo de las células tumorales podrían beneficiarse del tratamiento con bortezomib, y que en otros estudios en los que el tratamiento con este fármaco no fue el esperado se debiese a que no se realizó una selección del perfil molecular previo de los pacientes.

Nuevos biomarcadores predictivos de resistencia a CDDP

Analizando conjuntamente los resultados obtenidos del desarrollo de esta tesis, podríamos decir que este trabajo se ha centrado en la búsqueda de marcadores que predigan la respuesta a CDDP en CNMP. Entre los dos bloques de trabajo, hemos demostrado que tanto el estado de activación de los componentes de la vía de IGF-IR/PI3K/AKT y del receptor de EGF junto con el estado de metilación del promotor de *IGFBP-3* tienen un papel muy importante en el desarrollo de resistencia a CDDP. Además, la ausencia o presencia de la fosfatasa MKP1 y el estado de activación del factor de transcripción NFκB son otros elementos complementarios y de gran implicación en el desarrollo de resistencia.

La metilación del promotor *IGFBP-3* ha sido correlacionada previamente con datos clínicos que indicaban una pobre prognosis en cáncer de próstata y ovario (Wiley et al., 2006, Perry et al., 2007), y en estadios tempranos en pacientes de CNMP (Chang et al., 2002a, Chang et al., 2002b). Nosotros presentamos la metilación del promotor de *IGFBP-3* como un biomarcador predictivo de respuesta a CDDP en CNMP, siendo la primera vez que se describe la metilación de un gen como un marcador de respuesta a un quimioterápico en este tipo tumoral. Hemos encontrado una buena correlación (80,5%) en muestras de pacientes de CNMP entre el estado del promotor de *IGFBP-3* y respuesta a CDDP. Con estos datos hemos realizado para la escritura de esta tesis un test de diagnóstico, obteniendo una exactitud de 0,80, una especificidad de 0,88, y un ratio de probabilidad positiva de 6,26.

Por ello, un seguimiento de los pacientes después del tratamiento con quimioterapia mediante un test no invasivo midiendo el estado de metilación de *IGFBP-3* en ADN tumoral circulante en fluidos corporales como sangre, fluido bronquioalveolar o saliva, podría identificar la aparición de células resistentes a CDDP, y así predecir la respuesta del paciente al siguiente tratamiento con CDDP antes de la aparición de recidivas. Además, es importante resaltar la existencia de agentes que revierten los cambios epigenéticos y que han mostrado resultados prometedores en el modelo de ratón de carcinogénesis de pulmón y que están siendo ensayados en pacientes de

cáncer de pulmón (Belinsky et al., 2003). Pero además hemos observado que los cambios que induce el CDDP sobre la expresión de *IGFBP-3* promueven resistencia al fármaco a través de la vía de señalización de IGF-IR/PI3K/AKT. Con estos datos hemos presentado un test predictivo que mejoraba al anterior, con el que a partir de muestras de pacientes, midiendo el estado de metilación del promotor de *IGFBP-3* y el estado de activación de IGF-IR, EGFR y AKT se podría predecir con una mayor exactitud (0,84), una mayor especificidad (0,9) y con un mayor ratio de probabilidad positiva (9,75) la posible resistencia a CDDP. Obviamente, es necesario evaluar este test con población adicional para probar su utilidad clínica en la selección de pacientes con alta probabilidad de respuesta a CDDP sólo o en combinación con terapias basadas en la inhibición de EGFR, IGF-IR o AKT.

Asimismo hemos demostrado que la activación de las vías de PI3K/AKT y NFκB llevan a una reducción de la sensibilidad a CDDP en líneas de CNMP y que la inhibición de la actividad de éstas aumenta la citotoxicidad del CDDP en células de CNMP que presenten bajos niveles de expresión de la fosfatasa MKP1. Por todo ello, proponemos a MKP1 como una nueva diana molecular para el tratamiento del CNMP y además, su expresión podría utilizarse como un biomarcador predictivo de respuesta a CDDP junto con AKT y NFκB activados. También hemos observado que las líneas celulares que presentan actividad del factor de transcripción NFκB son sensibles al tratamiento con bortezomib y aunque en diferentes ensayos clínicos se ha observado que este fármaco no presenta buenos resultados en el tratamiento de CNMP, esto pudo deberse a que no hubo una selección molecular previa de los pacientes a tratar basada en los niveles nucleares de p65/RelA o RelB.

Con este trabajo hemos profundizado en el conocimiento global de las rutas de activación de respuesta a CDDP y su implicación en resistencia al fármaco. Existen una gran variedad de rutas que se activan o se inhiben en respuesta a CDDP, y esto ocurre de diferente manera en las distintas líneas de CNMP.

Por ejemplo, habíamos observado en la primera parte del trabajo que la línea celular de CNMP H460 no presentaba metilación del promotor de *IGFBP-3* ni una activación basal de AKT, por lo que otras vías de señalización debían de estar implicadas en la respuesta a CDDP. Posteriormente, hemos observado que en esta línea celular la fosfatasa MKP1 juega un papel muy importante en la sensibilidad al quimioterápico. Igualmente, vemos como en la línea A549 no se observan alteraciones epigenéticas en cuanto al promotor del gen *IGFBP-3,* y aunque determinar la activación del IGF-IR no fue posible, observamos que no existía activación de AKT; no obstante, su papel tumorogénico podría estar mediado por la presencia que observamos tanto de MKP1 como por la activación de la ruta de NFκB.

Analizando conjuntamente los datos obtenidos con las líneas celulares de CNMP ensayadas en ambas partes de la tesis, observamos que si tenemos en cuenta sus IC_{50}, la metilación del promotor de *IGFBP-3,* la fosforilación de AKT e IGF-IR, la expresión de la proteína MKP1 y la activación del factor de transcripción NFκB observamos que las células más resistentes, como H727 y H1299, presentaban al menos cuatro de los cinco parámetros observados. Y que la línea más sensible a CDDP, H460, sólo presentaba niveles altos de MKP1 (Tabla 3).

Con esto queremos resaltar que la respuesta a fármacos es un mecanismo complejo y muy diferente de unas líneas celulares a otras. Esta heterogeneidad se puede extrapolar a pacientes de CNMP. Por ello un estudio molecular de las diferentes vías en estas muestras de biopsias de pacientes podría mejorar la respuesta a los distintos tratamientos, posibilitando un tratamiento personalizado.

LÍNEAS CELULARES	IC_{50} (µg/ml)	*IGFBP-3*	AKT	IGF-IR	MKP1	NFκB
H727	9	M	*	_	*	*
H1299	6	M	*	*	_	*
A549	2	NM	_	¿?	*	*
H23	0,52	SM	*	*	_	*
H460	0,29	NM	_	_	*	_

Tabla 3. Descripción de los diferentes biomarcadores estudiados en este trabajo en relación al índice de resistencia que presentan cinco líneas celulares de CNMP. La combinación del estado de metilación del promotor de *IGFBP-3,* los cambios en la fosforilación de AKT, IGF-IR y NFκB y los niveles proteicos de MKP1 pueden predecir la respuesta al quimipterápico CDDP en líneas tumorales de CNMP. M=metilado, NM=no metilado, Asterisco =activación o expresión, Guión=no activación o expresión.

CONCLUSIONES

1. A partir de la combinación de las técnicas de microarrays de expresión y el tratamiento de reactivación epigenética en los grupos de líneas tumorales de CNMP sensibles/resistentes a CDDP, identificamos entre 9 genes seleccionados, el gen *IGFBP-3*, cuya validación epigenética posterior confirma la presencia de metilación en su promotor específica de resistencia a CDDP.

2. La metilación de Novo que sufre el gen *IGFBP-3* es causa y no consecuencia del proceso de resistencia en seis líneas celulares tumorales humanas, ya que tanto la inhibición de su expresión en las líneas sensibles, como su reexpresión en las líneas celulares resistentes, modifica la respuesta en viabilidad a CDDP. Además existe una relación directa entre la metilación del promotor de *IGFBP-3* y la resistencia a CDDP analizada en 23 líneas celulares tumorales adicionales.

3. La respuesta a CDDP mediada por la metilación del promotor de *IGFBP-3* se produce a través de la vía de señalización de IGF-IR/PI3K/AKT. Los cambios en la expresión del gen *IGFBP-3* modifican la activación tanto de AKT como de IGF-IR, induciendo cambios en la sensibilidad celular a CDDP en diferentes líneas celulares de CNMP, cáncer de ovario y páncreas, indicando un mecanismo general de resistencia en diferentes tipos tumorales. Además, diversos ensayos funcionales confirman que AKT es la responsable en parte de la resistencia adquirida a CDDP en estas células.

4. La aplicación traslacional de estos resultados demuestran una correlación significativa entre la metilación del promotor de *IGFBP-3* y la resistencia a CDDP en las muestras de CNMP analizadas. Asimismo, el seguimiento clínico de estos pacientes indica que aquellos diagnosticados en estadio I y que no tienen metilado el promotor de *IGFBP-3,* presentan una clara mayor supervivencia libre de enfermedad, comparado con otros grupos.

5. La metilación del promotor de *IGFBP-3* y la fosforilación de IGF-IR y AKT ocurre sólo en pacientes resistentes a CDDP, indicando que la vía de señalización de IGF-IR/PI3K/AKT también podría mediar la respuesta al fármaco en estos pacientes. Estos resultados permiten presentar un test predictivo de respuesta a CDDP de alta exactitud y especificidad, que considera necesario que una muestra presente al menos dos modificaciones de estas cuatro: la metilación del promotor del gen *IGFBP-3,* la fosforilación de IGF-IR, la fosforilación de EGFR y la fosforilación de AKT, para obtener un verdadero cambio en el fenotipo en términos de respuesta a CDDP.

6. En líneas celulares de CNMP tanto la vía de señalización de PI3K/AKT, como la del factor de transcripción NFκB y la expresión de la fosfatasa MKP1 están implicadas en la respuesta a CDDP. Observamos que la inhibición de la activación de AKT como de la vía de NFκB sensibilizan a las células al tratamiento con CDDP, pero sólo cuando existen bajos niveles de MKP1 o inhibimos artificialmente su expresión.

7. Los factores de transcripción NFκB, p65/RelA y RelB se localizan frecuentemente en el núcleo de las muestras de pacientes de CNMP analizadas. El inhibidor del proteasoma, bortezomib, inhibe el crecimiento de las células de CNMP con alta actividad trancripcional dependiente de NFκB, indicando que aquellos pacientes cuyos tumores presenten p65/RelA y RelB localizadas en el núcleo celular podrían beneficierse del tratamiento con bortezomib.

8. Por último, con los resultados conjuntos de esta tesis, hemos profundizado en el conocimiento de los diferentes mecanismos y rutas celulares implicados en el complejo proceso de resistencia a CDDP, permitiendo presentar diversos biomarcadores que pueden ser complementarios prediciendo la respuesta a este quimioterápico y que podrían ser utilizados para un tratamiento personalizado en pacientes con CNMP, resultando en un mayor beneficio clínico.

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ANEXO I

Artículos que no forman parte de la tesis

Artículos publicados durante el desarrollo de la tesis que no pertenecen a la misma:

MicroRNAs as novel epigenetic biomarkers for human cancer.
Cortés-Sempere M, Ibáñez de Cáceres I.
Clin Transl Oncol. 2011 Jun;13(6):357-62.

Mitogen-activated protein kinase phosphatase-1 in human breast cancer independently predicts prognosis and is repressed by doxorubicin.
Rojo F, González-Navarrete I, Bragado R, Dalmases A, Menéndez S, **Cortes-Sempere M,** Suárez C, Oliva C, Servitja S, Rodriguez-Fanjul V, Sánchez-Pérez I, Campas C, Corominas JM, Tusquets I, Bellosillo B, Serrano S, Perona R, Rovira A, Albanell J. Clin Cancer Res. 2009 May 15;15(10):3530-9. Epub 2009 May 5.

The role of the NFkappaB signalling pathway in cancer.
Cortés Sempere M, Rodríguez Fanjul V, Sánchez Pérez I, Perona R.
Clin Transl Oncol. 2008 Mar;10(3):143-7. Review.

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MOLECULAR TARGETS IN ONCOLOGY

microRNAs as novel epigenetic biomarkers for human cancer

María Cortés-Sempere · Inmaculada Ibáñez de Cáceres

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Abstract MicroRNAs (miRNAs) are regulatory, non-coding RNAs that are approximately 22 nucleotides in length. Nearly 1000 unique miRNAs encoded in the human genome have been identified, shedding new light on the post-transcriptional regulation of more than one-third of human genes. These miRNAs are involved in numerous biological processes, including development, differentiation, apoptosis, homeostasis and stem cell biology. Aberrant miRNA expression patterns also play a substantial role in carcinogenesis. It is believed that genetic and epigenetic regulation is responsible for changes in miRNA expression in cancer development, however the exact mechanisms remain unclear. miRNAs are involved in almost all aspects of cancer biology such as apoptosis, invasion, metastasis and angiogenesis. Thanks to this wide range of biological functions, the analysis of changes in overall miRNA expression occurring within human tumours has helped identify miRNA signatures associated with diagnosis, staging, progression, prognosis and response to treatment. This positions miRNA-targeting therapeutics as a novel and promising tool for cancer treatment.

Keywords MicroRNA expression profile · Epigenetics · Tumour suppressor genes

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Introduction

MicroRNAs (miRNAs) are evolutionarily conserved, small RNAs that repress gene expression at the post-transcriptional level. Release 17 (April 2011) of the miRBase database (http://www.mirbase.org/index.shtml) encompasses 19,724 mature miRNA products, in 153 species. The number of reported human miRNAs is in excess of 900, five times as many as initial calculations indicated [1]. miRNAs are named as miR-number (e.g., miR-103). An additional letter is included to distinguish miRNAs of similar sequences (e.g., miR-103a) and an extra number is added to identify miRNA of identical mature sequences (usually with different precursor sequences) that could be encoded at several genomic loci (e.g., miR-103a-1, miR-7-1). miRNAs are usually located in the intronic region of protein-coding or non-coding transcription units [2], although they can also be derived from genomic repeat sequences [3]. They are synthesised by an RNA polymerase II as a pri-miRNA with a 5' cap structure and 3' poly (A) tail [4]. Pri-miRNA is then processed to pre-miRNA by the RNase III Drosha in the nucleus [5]. Pre-miRNA is exported to the cytoplasm by exportin-5 [6], where it is converted to a mature miRNA duplex by the RNase III Dicer [7]. Generally, only one strand is selected as the biologically active mature miRNA and the other strand is degraded. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) and drives the selection of target mRNAs containing antisense sequences. In terms of the degree of complementarity between the miRNA and the match mRNA sequences, the mRNA will be degraded and/or the protein translation process will be inhibited [8, 9]. However, miRNAs downregulate most of their endogenous targets by less than 50%, even when the specific miRNA is overexpressed [10]. Thus, after this level of inhibition, most proteins should remain active, suggesting that although most genes are predicted to be miRNA targets, only

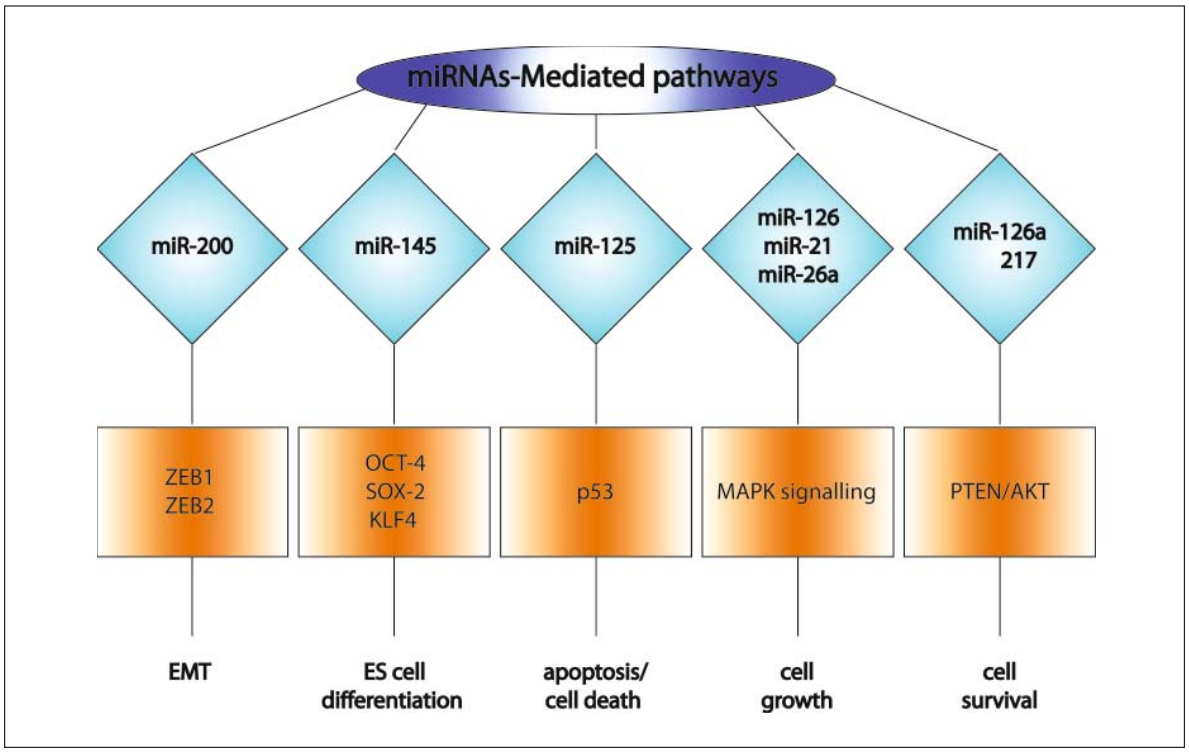

Fig. 1 Example of cell biological pathways in which the regulation of listed miRNAs acting though specific mRNA gene-proven targets has been reported

some will prove miRNA-intrinsic regulation for biological responses. Some of the best known cellular pathways considered prime candidates for miRNA-mediated regulation in animal cells are signal transduction pathways such as TGF-ß, ES differentiation or MAPK signalling pathways between others (Fig. 1) [11]. A possible way of identifying the biological processes in which miRNA is involved is to combine array genomic hybridisation data and miRNA expression data obtained from the comparison of two different biological situations, e.g., normal and tumour cell lines.

MicroRNAs and human cancer

The first evidence correlating miRNA and cancer appeared in 2002 when it was observed that miR-15a and mi-R-16-1 were clustered at a frequently deleted region at Cr13, in B-cell chronic lymphocytic leukaemia [12]. Currently, we know that miRNAs are aberrantly expressed or mutated in a variety of cancers, suggesting that they are involved in the initiation and progression of human cancer. Depending on their targets, miRNAs could act as oncogenes or tumour suppressor genes: miR-17-92, miR-372, miR-373 and miR-155 have been reported to act as oncogenes in lymphoma, lung cancer, testicular germ cell tumours and B-cell lymphoma [13, 14], while Ras and HMGA2 [15, 16] are suppressed by let-7 in lung cancer. Bcl-2 is suppressed by mir-15 and mir-16 in leukaemia [17], and miR-17-5p and miR-20a control the cell-death/proliferation driven by c-Myc [18]. This aberrant expression of key miRNAs regulating important oncogenes, such as Ras, Bcl-2 and Myc, and TSGs such as p53 or PTEN, can disturb the balance between cellular growth and cellular suppression, thus causing the possible generation of tumour cells.

Classifying human cancers using miRNA expression profiles

The introduction of microarray techniques has facilitated the analysis of changes that occur in miRNA expression in human cancer. In addition to differentiating tumours from normal tissue, miRNA expression profiles may also reflect the origin and differentiation state of tumours and identify the progression, prognosis and even the response to treatment [19]. Thanks to an increasing number of systematic analyses of cancer samples, miRNA expression profiles have been shown to be better predictors of cancer type and stage than mRNA expression profiles, making them a useful tool for cancer diagnosis and prognosis [20]. Remarkably, expression-profiling experiments have shown a global

downregulation of miRNAs in tumour samples compared with normal tissues, although some of them are increased [21, 22]. Although there are some miRNAs, such as miR-143 and miR-145, that have been reported to be aberrantly expressed in several different cancer types [23–25], the miRNA signatures of cancers of different cellular origin seem to be unique. Describing all miRNAs that have been reported to change in all tumour types is beyond the capacity of this review and so this report will instead provide some cancer-specific profiling data.

Ovarian cancer
A small set of 29 miRNAs, four of which are up-modulated (miR-200a, miR-200b, miR-200c and miR-141) and 25 of which are down-modulated (with miR-199a, miR-140, miR-145 and miR-125b1 among the most down-modulated), seems to be a miRNA expression signature that is able to discriminate ovarian cancer tissues from normal ovaries, with a classification rate of 89% [26].

Breast cancer
miRNA expression profiles have also been used for the molecular characterisation of circulating tumour cells (CTC) in a cohort of 50 metastatic breast cancer patients [27]. This study resulted in various patient clusters that ended with ten CTC-specific miRNAs, including miR-183, -184, -379 and -424.

Lung cancer
The various histologic subtypes for non-small-cell lung cancer may also be discriminated, particularly in early stages, using miRNAs located on loci that are often altered in lung cancer (3p21-22), such as the let-7 family, miR-26a and miR-98 [28]. The authors also found a five-miRNA signature that significantly predicted survival for squamous cell carcinoma (miR-181a, -191, -107 and -103).

Prostate cancer
A panel of 51 miRNAs, identified by comparing benign and malignant tumours, 37 of which showed down-regulation and 14 up-regulation in malignant samples, may be significant in prostate cancer development [21]. It has recently been reported that miR-143 and miR-145 are involved in the regulation of MYO6 expression and possibly in the development of prostate cancer [29].

Epigenetic regulation of tumour-suppressor-miRNAs

A major gap in our knowledge of miRNAs relates to their expression mechanisms. The causes of differential expression of miRNAs in malignant cells when compared with normal cells may be explained by the location of these genes in cancer-associated genomic loci, by alterations in the miRNA processing machinery and/or by epigenetic mechanisms. Several studies have identified the presence of genomic abnormalities in miRNA genes [19, 30]. More recently, mounting evidence has shown that miRNA genes are also regulated by epigenetic mechanisms. Promoter regions of miRNAs, which are susceptible to epigenetic regulation, are beginning to be unmasked either through *in silico* studies that predict promoter regions, which in most cases have not been confirmed [31], or with proven laboratory experiments that have identified independent promoters within the intron where the miRNA is embedded, as is the case with miR-34 [32]. A novel mechanism was reported three years ago for silencing intronic tumour suppressor miRNAs in cancer through epigenetic changes that directly control their host genes. This was the case for miR-342, in which epigenetic reactivation not only re-expressed miR-342, but also its host gene EVL [33]. This mechanism has been also reported for miR-126 [34].

Those miRNAs under epigenetic regulation have been identified using various approaches: firstly, searching for CpG islands near miRNA-encoded regions turned up dozens of candidates such as mi-RNA-9, -124 or -148a [35]. A second approach that looked for upregulated miRNAs after DNA methyltransferase (DNMTs) mutation identified miR-124 [36]. A third strategy, using either combined or individual treatment with demethylating and/or histone deacetylase inhibitor agents, is resulting in a clearer identification of *bona fide* miRNAs, such as miR-127, -34 and -203, under epigenetic regulation in cancer development [37–39]. The initial report by Saito et al. [38] established that the expression of miR-127 is regulated epigenetically and demonstrated that it has tumour-suppressor properties. In this study, pharmacological unmasking of epigenetically silenced miRNAs activated 17 of 313 miRNAs analysed in the bladder cancer cell line T24 and the normal fibroblast cell line LD419. Aberrant promoter methylation and histone modification status of miR-127 correlated significantly with mature miR-127 expression. In the last 5 years, the number of studies documenting the epigenetic regulation of miRNAs has increased dramatically. It is beyond the capacity of this review to describe all miRNAs found to be under epigenetic regulation in cancer development, therefore some of the best-known miRNAs implicated in this process have been selected (Table 1).

miR-124 is represented in three genomic loci, miR-124-1 (8p23.1), miR-124-2 (8q12.3) and miR-124-3 (20q13.33), and is the most abundant miRNA in the adult brain. However, epigenetic silencing of the three loci is a frequent event not only in brain tumours but also in different tumour types such as colon, breast and leukaemia [36, 37, 40, 41]. miR-124 targets the oncogene CDK6, therefore miR-124 epigenetic silencing activates CDK6 expression, which phosphorylates Rb and results in a cell growth burst. DNA demethylation drugs could revert this phenotype [36]. A strong association between increased miR-124 methylation levels and the presence of *Helicobacter pylori* infection in gastric mucosa has also been reported, an infection related to gastric carcinogenesis [42]. miR-137 also targets the 3' UTR of *CDK6* and is surrounded by a CpG island that is specifically methylated in several types of cancer [43, 44].

Table 1 Tumour distribution and laboratory-proved target genes for some representative miRNAs under epigenetic control

miRNA genes	Gene methylation-related tumour	Known target genes	Reference
miR-9	Breast, renal cell carcinoma, leukaemia	NFKB1	[35, 41, 75]
miR-34	Colorectal, leukaemia, oral, gastric	CDK6, MYC, CREB	[32, 39, 44, 76]
miR-124	Colorectal, breast, leukaemia, hepatocellular carcinoma	CDK6	[36, 37, 40]
miR-126	Bladder, prostate cancer	SpReD1, pIK3R2	[34]
miR-127	Bladder	BCL6	[38]
miR-137	Oral, colorectal	CDK6, E2F6	[43, 44]
miR-145	Prostate cancer	IRS-1	[50]
miR-148	Breast cancer	TGIF2	[35]
miR-200	Pancreatic, lung, bladder	ZEB1, ZEB2, SOX2, KLF4	[47–49]
miR-203	Hepatocellular carcinoma, gastric	ABL1, BCR-ABL1	[37, 77, 78]
miR-342	Colorectal	DNMT-1	[33]
miR-375	Breast, gastric	JAK1, PDK1	[79, 80, 81]
let-7a	Ovarian, lung cancer	IGF2BP1-3	[51, 82]

The miR-200 family (miR-200a, -200b, -200c and -141) has been shown to regulate epithelial-mesenchymal transition (EMT) and cell migration in a variety of cancer cell lines through the promotion of E-cadherin by directly targeting the transcription factors ZEB1 and ZEB2 [45]. Recently, promoter hypermethylation of miR-200c has been found to be responsible for the loss of miR-200c in invasive non-small-cell lung cancer cells and in bladder cancer [46, 47], and also reported to be an early manifestation during carcinogen-induced transformation of human lung cancer cells [48]. However, miR-200a and miR-200b have been reported to be hypomethylated and overexpressed in pancreatic cancer [49].

MiR-145 was significantly downregulated in various types of human cancer cell lines and primary samples [50]. This downregulation is mediated through both DNA promoter hypermethylation and p53 mutation status. The authors found a significant correlation between miR-145 expression and the status of the p53 gene, based on an electrophoretic mobility shift assay showing that p53 binds to the p53 response element upstream of miR-145, but the binding was inhibited by hypermethylation.

The let-7 family of miRNAs is aberrantly downregulated in breast and lung tumours, leading to RAS pathway oncogenic activation [48, 51]. However, let-7a-3 also has oncogenic potential; indeed, hypermethylation at the let-7a-3 locus in ovarian cancer correlates with low IGF-II levels via targeting IGFBP1 and 2 and with a favourable prognosis [52].

Interestingly, cancer cells may also use miRNAs to neutralise the CpG island methylation that is involved in the initiation and progression of cancer, acting on DNMTs as targets, as has been reported with miR-29 and miR-148 that directly target DNMT-3A and/or -3B [53–55], and with miRNA-342 that targets DNMT-1 [56]. Thus, re-expression of miR-29 results in an overall reduction in the number and size of tumours in mice [54]. Also the re-expression of miR-143, which also controls DNMT3A expression, reduces tumour cell proliferation *in vitro* colon cancer cells [57].

miRNAs as biomarkers and targets for cancer therapy

miRNA expression clearly differs between healthy and malignant tissue, with oncogenes and tumour suppressor genes their mRNA targets. Therefore miRNA may offer effective tools for cancer diagnosis and therapeutics. For some types of cancer, the possibility of detecting specific miRNAs in serum has been reported, as is the case for miR-155, -210 and -21 from B-cell lymphoma patients [58], or miR-34a and -155 from metastatic breast cancer patients [59]. In terms of RNA-based therapeutics, instead of focusing on protein-coded oncogenes, which are difficult to target therapeutically, one could focus on their target miRNA. Manipulation of miRNAs includes overexpression and inhibition of their expression. The use of synthetic miRNA (mimics) and antisense oligonucleotides (antagomirs) to overexpress and knockdown miRNAs has been reported [60, 61]. These anti-miRNA therapies could represent well designed drugs that could potentially inhibit tumour growth and progression. In addition, there has been a recent proposal for overexpressing the miRNA target sequence, applied as a competitive inhibitor by sequestering the miRNA and inhibiting its function over the endogenous target [62].

From the multitude of *in vitro* studies conducted so far, miRNAs such as miR-126, miR-155, miR-122, miR-21, let-7, miR-29, miR-30 and miR-221/222 appear to be candidates for focusing the development of therapeutics. miR-126 has been proposed as a good target for anti-angiogenic therapies, as it is the most highly enriched miRNA in endothelial cells, promoting angiogenesis and vascular integrity by targeting SpReD1 and pIK3R2 mRNAs [63, 64]. miR-155 knockdown rendered breast cancer cells sensitive to chemotherapy through regulation of FOXO3a [65]. miR-21 is an oncogenic miRNA overexpressed in various tumour types including colon, breast and lung [24, 66, 67]. MiR-21 targets the mRNA from various TSGs, e.g., PTEN or BTG2 [68, 69]. Knockdown-induced apoptosis restores the normal proliferation rate in human glioblastoma cells [70]. Therefore, a direct therapy against miR-21 may be an

important strategy that could be used for a wide range of tumours.

The role of miRNA in regulating tumour drug response is also supported by several studies conducted *in vitro*. Two large, separate studies have examined overall miRNA expression in the NCI-60 cell panel, first under exposure to 3089 compounds, and second after integration of miRNA and mRNA expression. These studies led to the identification of miRNAs that significantly correlate with drug potency and sensitivity [71, 72]. Results from these studies clustered the miRNA according to both malignancy of origin in certain malignancies and by drug response. For example, miR-21 has been implicated in gemcitabine resistance in pancreatic cancer [73], while miR-92-a-2*, miR-147 and miR-574-5p were associated with chemoresistance in small-cell lung cancer [74].

In conclusion, identification and understanding of the miRNA/mRNA-target relationship will help us gain insight into exactly how miRNAs play their role in cancer establishment and progression. The next step would be to link this knowledge to form a cancer-specific network, as many miRNAs target the same biological cell pathways. Once we achieve this level of knowledge, the use of traditional therapies with current drugs that target specific cellular pathways in combination with miRNA-directed therapy could open a new era of personalised treatments for cancer patients.

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Conflict of interest The authors declare that they have no conflict of interest relating to the publication of this manuscript.

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Clinical Cancer Research

Mitogen-Activated Protein Kinase Phosphatase-1 in Human Breast Cancer Independently Predicts Prognosis and Is Repressed by Doxorubicin

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Cancer Therapy: Preclinical

Mitogen-Activated Protein Kinase Phosphatase-1 in Human Breast Cancer Independently Predicts Prognosis and Is Repressed by Doxorubicin

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Abstract

Purpose: Mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) dephosphorylates mitogen-activated protein kinase [extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38], mediates breast cancer chemoresistance, and is repressible by doxorubicin in breast cancer cells. We aimed to characterize doxorubicin effects on MKP-1 and phospho-MAPKs in human breast cancers and to further study the clinical relevance of MKP-1 expression in this disease.

Experimental Design: Doxorubicin effects on MKP-1, phospho-ERK1/2 (p-ERK1/2), phospho-JNK (p-JNK), and phospho-p38 were assayed in a panel of human breast cancer cells by Western blot and in human breast cancer were assayed *ex vivo* by immunohistochemistry ($n = 50$). MKP-1 expression was also assayed in a range of normal to malignant breast lesions ($n = 30$) and in a series of patients ($n = 96$) with breast cancer and clinical follow-up.

Results: MKP-1 was expressed at low levels in normal breast and in usual ductal hyperplasia and at high levels in *in situ* carcinoma. MKP-1 was overexpressed in ~50% of infiltrating breast carcinomas. Similar to what was observed in breast cancer cell lines, *ex vivo* exposure of breast tumors to doxorubicin down-regulated MKP-1, and up-regulated p-ERK1/2 and p-JNK, in the majority of cases. However, in a proportion of tumors overexpressing MKP-1, doxorubicin did not significantly affect MKP-1 or phospho-MAPKs. With regard to patient outcome, MKP-1 overexpression was an adverse prognostic factor for relapse both by univariate ($P < 0.001$) and multivariate analysis ($P = 0.002$).

Conclusions: MKP-1 is overexpressed during the malignant transformation of the breast and independently predicts poor prognosis. Furthermore, MKP-1 is repressed by doxorubicin in many human breast cancers.

There is a need to find novel targets to improve the therapeutic options for breast cancer patients (1, 2). A recently proposed target is the mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1; refs. 3, 4). MAPKs, the substrates of MKPs, play important roles in proliferation, stress responses, apoptosis, and immune response (3–7). There are three well-known MAPK subfamilies: extracellular signal-regulated kinases (ERK), c-Jun NH_2-terminal kinases (JNK), and p38 MAPK isoforms. MAPKs are activated through a cascade of sequential phosphorylation events. The phosphorylation of MAPKs on threonine and tyrosine residues by specific upstream MAPK kinases (MEKs or MKKs) leads to their activated state.

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Translational Relevance

There is increasing evidence on a role of the mitogen-activated protein kinase phosphatase-1 (MKP-1) as a mediator of *de novo* or acquired breast cancer resistance in human cell lines and the search of MKP-1 inhibitors is actively pursued. Here, we provide evidence on MKP-1 biology in human breast cancer at three levels: (*a*) MKP-1 is overexpressed during the malignant transformation of the breast, (*b*) MKP-1 overexpression is linked with poor patient outcome, and (*c*) MKP-1 is a repressible enzyme by doxorubicin in many human breast cancers. This repression is associated with an increase in the levels of phospho-extracellular signal-regulated kinase 1/2 and c-Jun NH_2-terminal kinase. These results add clinical support to the concept of MKP-1 as a novel target for breast cancer therapy and justify further studies of MKP-1 as a promising prognostic marker.

Conversely, MKPs, also known as dual-specificity phosphatases, dephosphorylate MAPKs on tyrosine and threonine residues (3, 5, 8). The prototypic member of the family, MKP-1 is an inducible nuclear phosphatase able to dephosphorylate ERK, JNK, and p38. MKP-1 is induced by many of the same stimuli that activate MAPKs, including growth factors and stress (5). Furthermore, MAPKs can increase MKP-1 protein activity in two distinct ways: firstly, MKP binding to its MAPK target causes a subtype-specific enhancement of its catalytic activity (9), and secondly, by phosphorylation by ERK, which inhibits MKP-1 degradation through the ubiquitin pathway (10). This mechanism is viewed as a feedback control to attenuate MAPK signaling (4, 5, 11, 12). MKP-1 seems to play an important role in tumorigenesis (4, 13, 14) and counterbalances the cytotoxicity of various anticancer drugs (4, 14–21). In this regard, anthracyclines, alkylating agents, taxanes, cisplatin, or proteasome inhibitors induce apoptosis in part by activation of the JNK pathway (15, 16). Notably, high levels of MKP-1 may dephosphorylate JNK and therefore limit the cytotoxicity of these agents (14, 16, 17, 19–24). Conversely, down-modulation of MKP-1 might be proapoptotic by facilitating a persistent JNK phosphorylation (4, 17).

Several reports suggest MKP-1 as a potential target in breast cancer. In breast cancer cells, MKP-1 was a significant mediator of chemoresistance to anthracyclines, alkylating agents, and taxanes (15, 18, 19). Proteasome inhibitors induce MKP-1 and this induction played an antiapoptotic role (16, 20–23). Dexamethasone also induced MKP-1 and limited paclitaxel cytotoxicity (18, 19). In contrast, anthracyclines decrease expression of MKP-1 (15, 24). This repression is involved in the potentiation of the cytotoxicity of alkylating agents by anthracyclines (15). Aplidin also down-modulates MKP-1 (25). Complementing preclinical data, MKP-1 is overexpressed in human breast cancers (26, 27). These observations justify an interest in MKP-1 inhibitors for cancer therapy (3, 4, 19, 28, 29).

Here, we aimed to further characterize MKP-1 and phospho-MAPK regulation by doxorubicin in breast cancer cells and in an *ex vivo* model (30, 31). We also analyzed the expression of MKP-1 in breast malignant transformation and in breast cancer patients with clinical follow-up.

Materials and Methods

Reagents, antibodies, and cells. Doxorubicin and Ro-31-8220 (both from Calbiochem; ref. 32) were purchased for use. Doxorubicin was freshly dissolved in water and Ro-31-8220 was dissolved in DMSO at stock concentrations of 10 mmol/L. Elite avidin-biotin complex method kit was from Vector Laboratories. Enhanced chemiluminescence detection kit was from Amersham Pharmacia Biotech. All tissue culture materials were from Life Technologies. The following antibodies were used: anti–phospho-ERK1/2 (p-ERK1/2; Thr^{202}/Tyr^{204}), anti-ERK1/2, anti-JNK, anti-p38 MAPK, and anti–phospho-p38 (p-p38) MAPK (all from Cell Signaling Technology). Anti–phospho-JNK (p-JNK; Thr^{183}/Tyr^{185}) from Promega and Cell Signaling Technology was used for immunohistochemical and Western blot analysis, respectively. To detect MKP-1, two antibodies were used: one for Western blot and one for immunohistochemical assays. Antibodies to detect expression of estrogen receptor, progesterone receptor, and HER2 (HercepTest) were purchased from Dako. HER2 amplification was assayed by HER2 fluorescence *in situ* hybridization pharmDx (Dako). The human breast cancer cell lines BT-474, SK-BR3, MDA-MB-468, MDA-MB-453, MDA-MB-231, and MCF-7 (from the American Type Culture Collection) were maintained as previously reported (9).

Real-time quantitative reverse transcription-PCR. Specific PCR primer combinations and fluorogenic probes (5′-FAM, 3′-TAMRA) for the target MKP-1 mRNA and housekeeping RNA (endogenous control, 18S, β-actin, and RPLPO) were purchased from Applied Biosystems. Total RNA was isolated using RNeasy kit (Qiagen) and reverse transcribed to cDNA using SuperScript reverse transcriptase (Invitrogen). cDNAs were combined with primers and probes specific for each gene of interest along with predeveloped Taqman Gene Expression Master Mix (Applied Biosystems) for the following genes: *MKP-1* (Hs-00610256-g1), human *β-actin* (Hs99999903_m1), human *18S* (Hs99999901_s1), and human *RPLPO* (large ribosomal protein PO; Hs99999902_m1). The PCR protocol was 50°C for 2 min and 95°C for 10 min followed by 50 cycles at 95°C for 15 s and at 60°C for 1 min. Negative controls were included and yielded no products. Real-time PCR analysis was carried out on an ABI-7500HT. C_t values were determined using SDS v2.2 software (Applied Biosystems) and compared using the C_t method.

Western blot analysis. Western blot analysis in cultured cells was done following as previously reported (20). Briefly, cells were washed in PBS and scraped, and whole-cell lysates were prepared. Frozen breast tumor samples were analyzed by Western blot, also as described previously (33).

***Cell viability assay* in vitro.** Cells were seeded into six-well plates at 3×10^5 per well and allowed to adhere overnight. Cells were then treated as indicated in Results. The dishes were incubated for 21 h before trypsinization and counting using trypan blue and/or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay (9).

Patient data and tissue specimens for clinicopathologic analysis. The study was approved by the ethics committee of the hospital and conducted following institutional guidelines. For clinicopathologic correlations and outcome analysis, retrospective samples were needed. To this end, formalin-fixed paraffin-embedded breast cancer samples obtained from surgical specimens from patients with biopsy-proven breast cancer were retrieved. Normal breast tissues, hyperplastic lesions, and *in situ* carcinomas from the same specimens were also assessed. In addition, selected paired formalin-fixed paraffin-embedded and frozen samples were obtained. Tumor-node-metastasis (TNM) staging was classified using the American Joint Committee on Cancer staging system for breast cancer (34). Histologic grades were defined according to Scarff-Bloom-Richardson modified by Elston criteria (35). Estrogen and progesterone receptors and HER2 status were determined by immunohistochemistry

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or fluorescence *in situ* hybridization (36). Clinical data and follow-up were obtained from review of patient's medical records. Disease relapse was considered as any primary, regional, or distant recurrence, as well as the appearance of a secondary tumor.

A tissue microarray was also constructed. Three tissue cores (1-mm diameter) were obtained from each specimen. In addition, 30 complete sections were assayed for same markers to correlate with observed tissue microarray expression. The tissue cores were precisely arrayed into a new paraffin block guided by a defined x-y position using a tissue microarray workstation (T1000; Chemicon) as reported in the literature (37).

***Exposure of human breast cancers to doxorubicin* ex vivo.** In addition to retrospective formalin-fixed paraffin-embedded specimens, fresh breast cancer specimens were obtained to add *ex vivo* doxorubicin and assess its molecular effects (30, 31). Tissue slices, which were not needed for diagnostic purposes, from primary breast tumors larger than 1.5 cm were obtained from surgical specimens of patients newly diagnosed with invasive breast cancer. One slide (control sample) was put into culture medium, and a second slice (treated sample) was put on the same culture medium plus doxorubicin at 5 μg/mL (38). Incubation was done in 24-well plates at 37°C in a constant atmosphere of 5% CO_2 for 24 h. At 24 h, specimens were fixed in 10% neutral-buffered formalin for 16 h and embedded in paraffin under vacuum conditions. A full report of this method will be reported separately. Specimens were then assayed by immunohistochemistry.

Immunohistochemistry. Immunostaining was done using 3-μm tissue sections, placed on plus charged glass slides. After deparaffinization in xylene and graded alcohols, heat antigen retrieval was done in buffered solutions: pH 9 EDTA-based buffer (Dako) was used to detect MKP-1, JNK, and p-JNK; pH 8 citrate-based buffer (Ventana) was used to detect ERK1/2, p-ERK1/2, p-p38, and p38. Endogenous peroxidase was blocked by immersing the sections in 0.03% hydrogen peroxide for 5 min. Slides

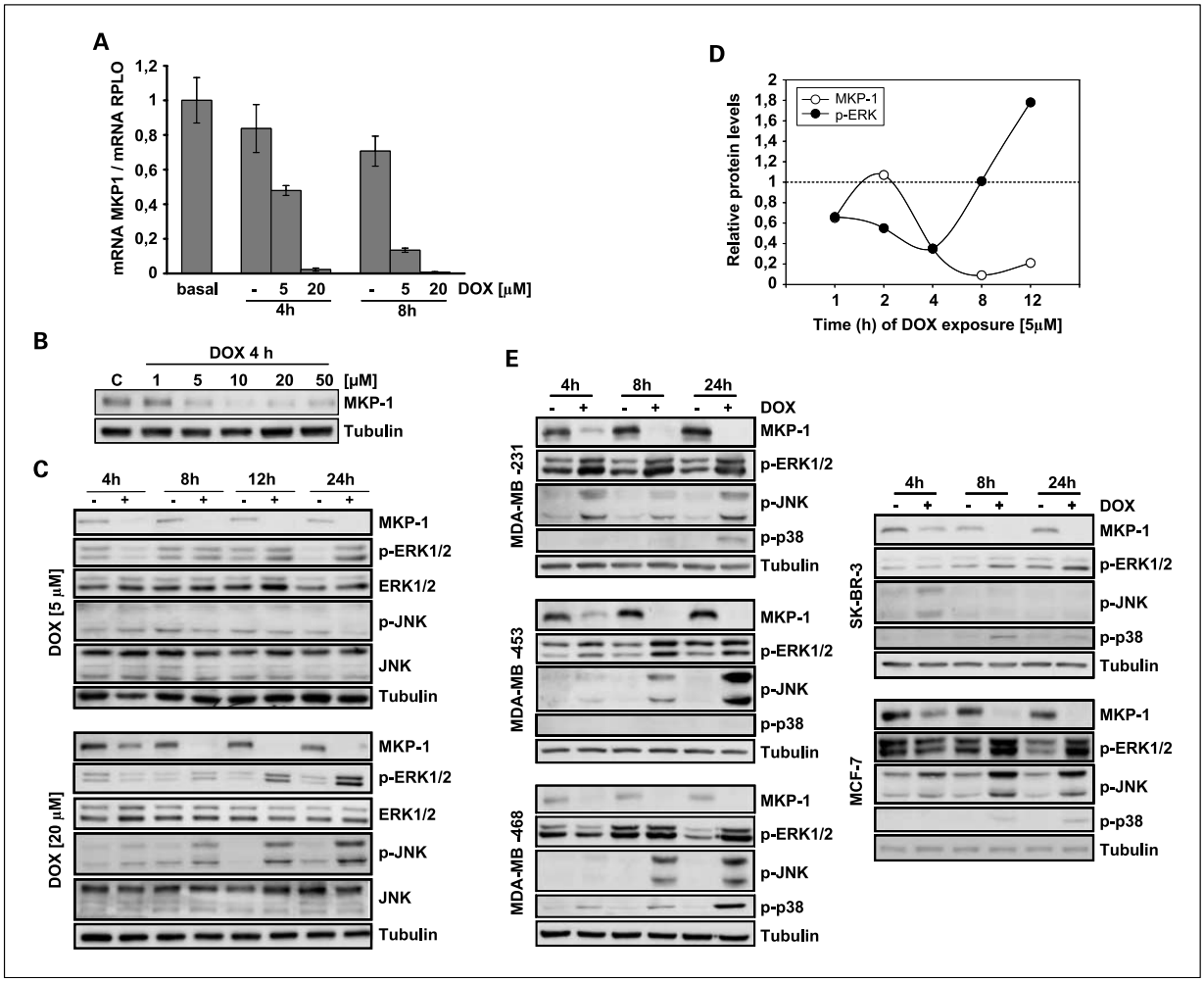

Fig. 1. Doxorubicin (*DOX*) effects on MKP-1 and phospho-MAPK expression in BT-474 breast cancer cells. *A,* levels of MKP-1 mRNA after doxorubicin exposure, measured by quantitative multiplex real-time reverse transcription-PCR. cDNA was synthesized from RNA samples from control- and doxorubicin-exposed cells. MKP-1 expression values are relative to the levels of a control RNA (RPLPO). Similar results were obtained with β-actin and 18S RNA. *B,* levels of MKP-1 protein in BT-474 cells exposed to increasing concentrations of doxorubicin, as assayed by Western blot. Tubulin was used as loading control. *C,* time course effects of doxorubicin (5 and 20 μmol/L) on MKP-1 and total and phosphorylated protein levels of MAPKs (ERK1/2 and JNK), as assayed by Western blot. No effects on p-p38 were seen (data not shown). Tubulin was used as loading control. *D,* graphic representation of a densitometric analysis of MKP-1 and p-ERK1/2 protein levels. *E,* time course effects of doxorubicin on MKP-1 and phospho-MAPKs (ERK1/2, JNK, and p38) in a panel of breast cancer cell lines, as assayed by Western blot. Tubulin was used as loading control.

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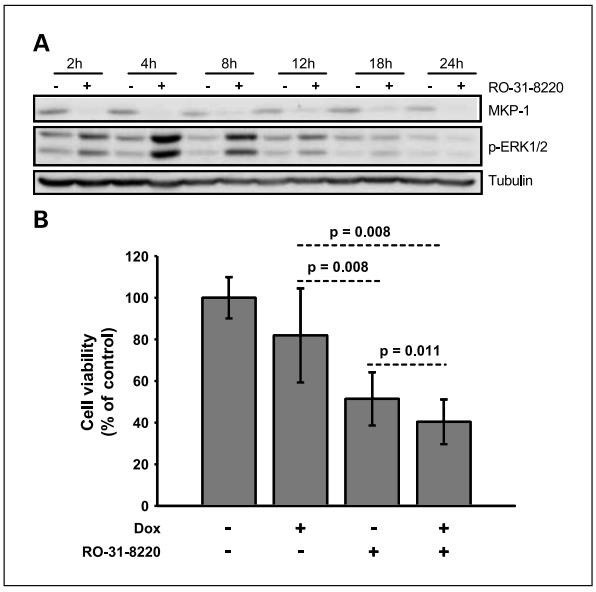

Fig. 2. *A*, time course of Ro-31-8220 (a nonspecific MKP-1 inhibitor) 3 h before treatment followed by a washout on MKP-1 and p-ERK1/2 in BT-474 cells, as assayed by Western blot. Please note that at 24 h the effects on p-ERK1/2 are reversed. *B*, effect of Ro-31-8220 before treatment on cell viability after 48 h of doxorubicin treatment, as assayed by manual trypan blue exclusion method.

were washed for 5 min with TBS solution containing Tween 20 at pH 7.6 and incubated with the primary antibodies for 60 min at room temperature followed by incubation with the appropriate anti-Ig horseradish peroxidase–conjugated EnVision polymer (Dako) or peroxidase-conjugated OmniMap multimer (Ventana) to detect antigen-antibody complexes. Sections were then visualized with 3,3′-diaminobenzidine as a chromogen for 5 min and counterstained with hematoxylin. All immunohistochemical stainings were done in Dako Link or Discovery XT platform and the same sections incubated with nonimmunized serum were used as negative controls. As positive control, sections of a breast human tumor with known marker expression were stained. In addition to human specimens, tissues (kidney and liver) for validation of MKP-1 immunohistochemical assay were obtained from wild-type and MKP-1 gene knockout mice (39), generously provided by Bristol-Myers Squibb Co., and processed in the same way.

Expression of markers was assessed in a blinded fashion by two investigators. For MKP-1 and MAPKs (ERK1/2, p-ERK1/2, JNK, p-JNK, p38, and p-p38), nuclear staining was required for considering a cell as positive. As we previously reported for phosphorylated forms of kinases, a tumor was scored as positive for a given phosphorylated protein when any percentage of tumor cells was stained (33, 40). In addition, a semiquantitative histoscore (Hscore) was calculated. The Hscore was determined by estimation of the percentage of tumor cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The formula used was Hscore = (low %) × 1 + (medium %) × 2 + (high %) × 3, and the results ranged from 0 to 300.

Statistics. Statistical analysis was carried out with Statistical Package for the Social Sciences version 13.0 (SPSS, Inc.). To analyze correlations between MKP-1 expression and clinicopathologic variables, we used the χ^2 test (Fisher's exact test) or Mann-Whitney test. A paired *t* test was used to compare molecular marker scores (immunohistochemistry) between control-treated and *ex vivo*–treated breast cancers. Effects of doxorubicin in breast specimens *ex vivo* were also expressed by means of unsupervised hierarchical clustering using the agglomeration rule average linkage that placed cases and marker results next to each other if they were most similar in their immunohistochemical profiles (41, 42). Disease recurrence was analyzed by the Kaplan-Meier method. Curves were compared by the log-rank test. Multivariate analysis, including continuous quantitative and qualitative clinicopathologic parameters, was done using the Cox proportional hazards model. All the statistical tests were conducted at the two-sided 0.05 level of significance.

Results

Doxorubicin affects MKP-1 and phospho-MAPK in breast cancer cells. We first assessed doxorubicin effects in BT-474 cells. As assayed by MTS at 48 hours, doxorubicin IC_{25} was ~5 μmol/L, IC_{50} was ~20 μmol/L, and IC_{75} was ~50 μmol/L in BT-474 (data not shown). A concentration- and time-dependent reduction of MKP-1 transcripts was observed on doxorubicin exposure, as assayed by reverse transcription-PCR (Fig. 1A) MKP-1 protein levels also decreased, with an evident effect at doxorubicin concentrations ≥5 μmol/L (Fig. 1B). MKP-1 protein down-regulation was observed at 4 hours (no effects were detected at earlier time points; data not shown) and was maximal at 8 hours (Fig. 1C). This inhibition was maintained at 24 hours, the latest time point tested. The decrease in MKP-1 preceded an increase in phosphorylated forms of MAPK family members. p-ERK1/2 expression increased in a time- and dose-dependent manner (Fig. 1C). Induction of p-JNK was observed at doxorubicin concentrations of ≥20 μmol/L (Fig. 1C, *bottom*, and experiments at 50 μmol/L not shown). p-p38 was undetected in BT-474 cells in all the conditions tested (data not shown). No evident changes were observed in total ERK1/2, JNK, and p38 proteins. A subtle reduction in p-ERK1/2 levels was observed at early time points (1-4 hours; Fig. 1D), which may decrease MKP-1 protein stability and perhaps contribute to MKP-1 down-modulation (10, 43). These kinetic effects were also observed at higher concentrations (data not shown). We next assayed five additional breast cancer cell lines (SK-BR3, MDA-MB-231, MDA-MB-468, MDA-MB-453, and MCF-7). In all of them, MKP-1 was down-modulated by doxorubicin and p-ERK1/2 and p-JNK were induced (Fig. 1E), as observed in BT-474 cells. In four of these cell lines, p-p38 was induced by doxorubicin (Fig. 1E).

We then combined doxorubicin with Ro-31-8220, an agent originally synthesized as a protein kinase C inhibitor, which reduces MKP-1 expression (32). A short exposure to Ro-31-8220 (3 hours followed by a washout) markedly reduced MKP-1 and induced p-JNK and p-ERK1/2. p-ERK1/2 levels returned to baseline at 24 hours (Fig. 2A). A combined treatment of doxorubicin and Ro-31-8220 resulted in a modest, but significant, additional reduction of cell viability compared with each agent used alone (Fig. 2B).

Validation of MKP-1 immunohistochemical assay and pattern of expression in normal and pathologic breast lesions. We tested the sensitivity and specificity of several anti–MKP-1 commercially available antibodies by immunocytochemistry (data not shown) and immunohistochemistry. A polyclonal anti–MKP-1 antibody was chosen based on results on tissues from wild-type and knockout MKP-1 mice (data not shown; ref. 39) as well as results in human tumor samples. Preabsorption with a specific antigen peptide resulted in a negative staining (data not shown). In addition, frozen human tumor samples assayed

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by Western blot had expression levels that corresponded closely to the levels observed by immunohistochemistry (data not shown).

We then assayed 30 complete tissue sections that included histologic normal breast (n = 30) as well as hyperplastic (n = 11), *in situ* (n = 18), and infiltrating (n = 30) malignancies (Fig. 3A). Histologically normal breast ductal and lobular epithelial cells exhibited diffuse and weak MKP-1 staining in the nuclei. In myoepithelial cells, MKP-1 was undetected and a low level of MKP-1 expression was seen in fibroblasts and endothelial cells. In usual ductal hyperplasia, the expression was similar to normal breast epithelium. All cases of benign breast tissue (normal and hyperplasia) had a MKP-1 Hscore <100. This Hscore value was selected to define the threshold of MKP-1 overexpression (Fig. 3B). In contrast, in all *in situ* carcinomas, MKP-1 was overexpressed (Fig. 3A and B). In infiltrating carcinoma, MKP-1 was overexpressed in up to 50% (Fig. 3A and B). In some specimens that had both *in situ* and infiltrating carcinoma, there was overexpression of MKP-1 in the *in situ* carcinoma, whereas it was not overexpressed in the infiltrating areas. Similar results in MKP-1 staining were observed in tissue microarray

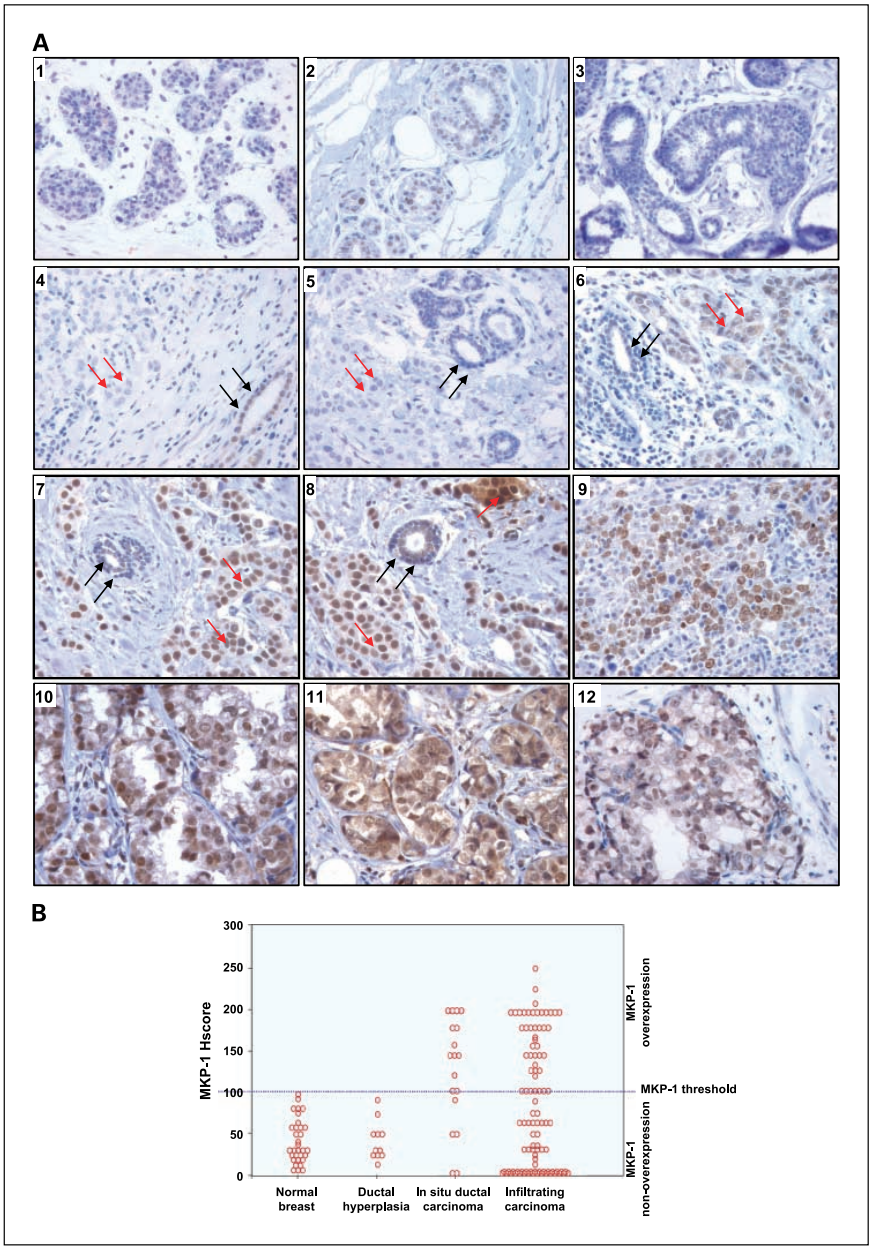

Fig. 3. *A,* representative pictures showing a representative range of MKP-1 expression levels observed by immunohistochemistry in human breast tissues. Histologically normal epithelial cells (*1* and *2*), as well as usual ductal hyperplasia (*3*), exhibited weak and diffuse nuclear MKP-1 staining. In infiltrating carcinoma cells, there was a wide range of expression levels, from undetected/low (*4* and *5*) to increasingly high MKP-1 staining (*4-11*). In pictures 4 to 8, infiltrating carcinoma areas are pointed by red arrows and adjacent histologically normal breast by black arrows. In pictures 9 to 11, only infiltrating carcinoma areas are shown. Picture 12 shows an *in situ* carcinoma with high MKP-1 expression. *B,* scatter plot by categories, displaying the levels of MKP-1 expression (Hscore) in normal breast, ductal hyperplasia, *in situ* ductal carcinoma, and infiltrating carcinoma.

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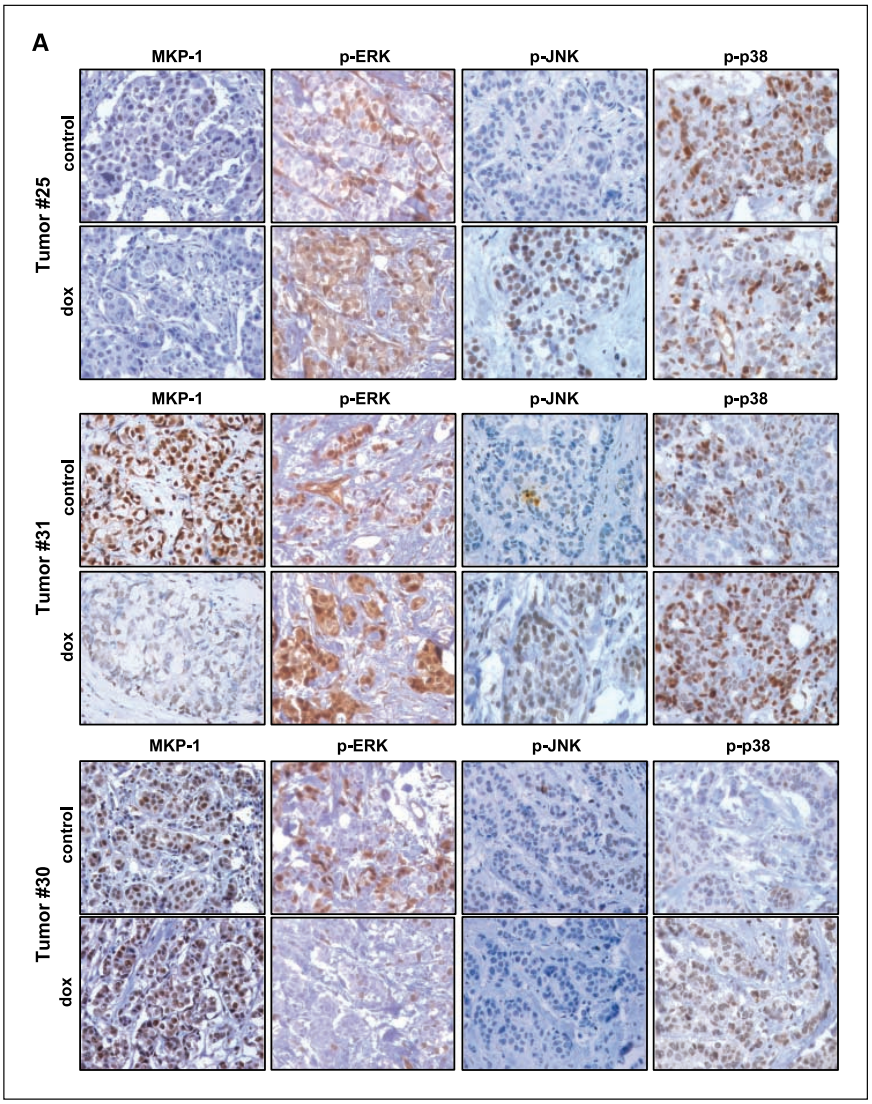

Fig. 4. Effects of doxorubicin, added *ex vivo* to fresh human breast cancer sections, on MKP-1 and phospho-MAPKs, as assayed by immunohistochemistry. *A,* three representative examples are shown. In tumor 25, doxorubicin resulted in an almost complete loss of detectable MKP-1 expression. p-ERK1/2 and p-JNK expression increased, whereas p-p38 expression was reduced by doxorubicin. In tumor 31, doxorubicin down-modulated MKP-1. p-ERK1/2, p-JNK, and p-p38 were up-regulated. In tumor 30, MKP-1 expression was slightly increased on doxorubicin exposure. p-ERK1/2 and p-JNK were down-regulated and p-p38 was up-regulated.

cores compared with the corresponding full tissue sections (data not shown).

Doxorubicin represses MKP-1 expression in a large subset of human breast cancers. Doxorubicin effects, as a single agent, on MKP-1 and phospho-MAPKs were assayed *ex vivo* in a series of 50 fresh breast cancer specimens under controlled experimental conditions (Fig. 4). In the whole series, doxorubicin induced a significant decrease in MKP-1 [mean Hscore MKP-1, 117 ± 64 (SD) in control versus 72 ± 104 in treated specimens; $P < 0.001$]. p-JNK and p-ERK1/2 expression increased significantly by doxorubicin (Hscore p-ERK1/2, 49 ± 47 in control versus 87 ± 65 in treated specimens; $P < 0.001$; Hscore p-JNK, 72 ± 50 in control versus 126 ± 82 in treated specimens; $P < 0.001$). p-p38 expression was numerically higher in doxorubicin-treated samples, but differences were not statistically significant ($P = 0.29$). Overall, this pattern (Fig. 4B and C) resembled the one observed in breast cancer cell lines (Fig. 1).

Effects, however, were not uniform; MKP-1 levels decreased on doxorubicin exposure in 39 specimens, whereas in the remaining 11 specimens the levels slightly increased (Fig. 4). Only in tumors that down-modulated MKP-1 levels there was a significant increase in p-ERK1/2 ($P < 0.001$) and p-JNK ($P < 0.001$). We then explored whether MKP-1 baseline levels might be linked to the ability of doxorubicin to down-regulate this protein. In the 27 tumors nonoverexpressing MKP-1, doxorubicin further down-modulated MKP-1 (MKP-1 Hscore, 69 ± 21 in control versus 13 ± 14 in treated specimens; $P = 0.03$). In this subset, a significant up-regulation of p-ERK1/2 and p-JNK was observed (p-ERK1/2 Hscore, 38 ± 44 in control versus 96 ± 71 in treated specimens; $P < 0.001$; p-JNK Hscore, 58 ± 44 in control versus 150 ± 65 in treated specimens; $P < 0.001$). In contrast, in the series of 23 tumors overexpressing MKP-1, doxorubicin did not significantly change MKP-1 expression ($P = 0.24$) and did not

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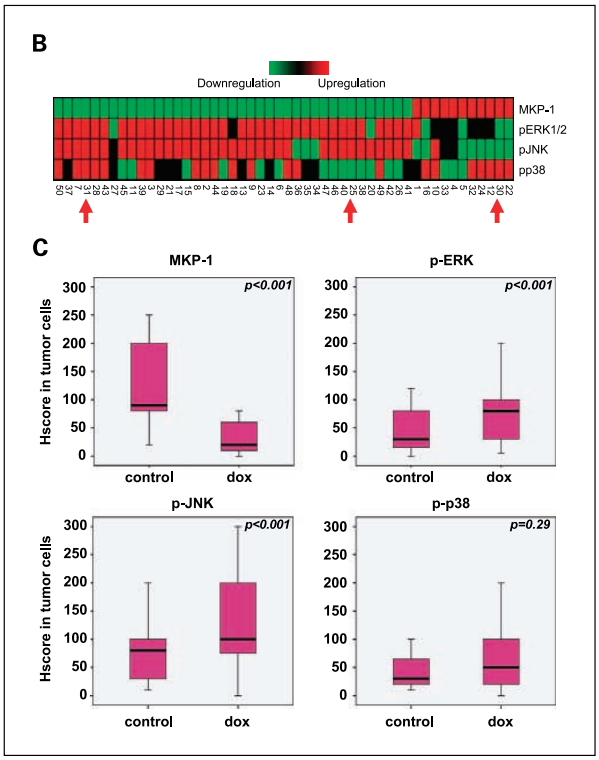

Fig. 4. *Continued. B,* hierarchical cluster analysis (41, 42) of doxorubicin effects on MKP-1 and phospho-MAPKs in a total of 50 human breast cancers. Each column represents an effect on expression of the paired control- and doxorubicin-treated samples, and each row, a single immunohistochemical marker, including MKP-1, p-ERK1/2, p-JNK, and p-p38. Down-regulation of expression is displayed in green, up-regulation in red, and no effect in black. Red arrows point the breast tumors displayed in *A. C,* box plot graphs indicating the immunohistochemical results for MKP-1 and phospho-MAPKs in control- and doxorubicin-treated conditions from *ex vivo* human breast cancers in the entire series ($n = 50$). The horizontal bar indicates median Hscore, the box represents the interquartile range, and the whiskers above and below the box show the minimum and maximum within a category. A *P* value for each marker is shown.

significantly affect p-ERK1/2 ($P = 0.23$), p-JNK ($P = 0.72$), or p-p38 ($P = 0.32$).

Expression of MKP-1, phospho-MAPKs, and clinicopathologic breast cancer features. Tumor specimens from paraffin-embedded blocks, with enough available tissue for the studies, were retrospectively selected from consecutive newly diagnosed breast cancer patients that had the following criteria: infiltrating ductal or lobulillar carcinomas, nonmetastatic disease at diagnosis, diagnosed between 1998 and 2000, and available clinical follow-up. A total of 96 patients with this criteria were selected (Table 1). There were 78 ductal and 18 lobulillar infiltrating carcinomas. Stage was I in 42 patients, II in 28 patients, and III in 20 patients (unknown in 6). Additional clinicopathologic characteristics are shown in Table 1. All patients had surgical treatment. Forty-four patients received systemic treatment (chemotherapy, antihormonal therapy, or both) according to our clinical guidelines (patients were not part of any clinical trial). Patients were followed at the medical oncology department (median follow-up, 69 months; range, 9-128). A total of 18 patients had a relapse.

Forty-three (45%) specimens overexpressed MKP-1 and 53 (55%) were nonoverexpressors (Fig. 3B). Mean MKP-1 Hscore was 85.3 (±79.5 SD). The following characteristics were numerically more frequent in tumors with MKP-1 overexpression: lobular infiltrating carcinoma, grade III, negative estrogen receptors, negative progesterone receptors, HER2 overexpression/amplification, large primary tumors, lymph node metastasis, and advanced TNM stage. However, none of these characteristics was significantly correlated with MKP-1 overexpression (Table 1). Twenty-four of 44 (54%) patients that received systemic treatment had MKP-1–overexpressing tumors, whereas in the 52 that did not receive adjuvant treatment, 17 (33%) had MKP-1 overexpression ($P = 0.16$). The more frequent use of adjuvant treatment in patients with MKP-1 overexpression may reflect that this was more common (albeit without reaching statistical significance) in patients with adverse prognostic features (Table 1). We also assessed the interplay between MKP-1 and phospho-MAPKs. All infiltrating breast cancers expressed some degree of p-ERK1/2, p-JNK, and p-p38. The mean Hscores and SD for these MAPKs were as follows: p-ERK1/2, 58.3 ± 58.7; p-JNK, 32.7 ± 47.7; and p-p38, 120.6 ± 76.7. Breast cancers overexpressing MKP-1 had slightly higher Hscore values of p-ERK1/2 (61.4 ± 54.2 in MKP-1 overexpressors versus 55.8 ± 62.4 in nonoverexpressors; $P = 0.020$) but had lower Hscore values of p-p38 (95.5 ± 71.6 versus 140.9 ± 75.4; $P = 0.005$). There were no significant differences in p-JNK (29.6 ± 29.7 versus 35.2 ± 41.6; $P = 0.98$) with regard to MKP-1 expression.

Overexpression of MKP-1 and breast cancer relapse. Disease-free survival analysis showed a higher risk of relapse in patients with MKP-1 overexpression (Fig. 5; $P < 0.001$, log-rank test). Kaplan-Meier survival curves for relapse and log-rank test comparisons also showed that high primary tumor stage ($P = 0.035$), tumor grade ($P = 0.013$), tumor size ($P = 0.025$), axillary lymph node involvement ($P < 0.001$), negative estrogen receptor status ($P = 0.008$), negative progesterone receptor status ($P = 0.004$), and low p-p38 expression ($P < 0.001$) were associated with a higher risk of relapse. A multivariate analysis was done including all the baseline clinicopathologic factors (Table 1) and MKP-1. In this analysis, MKP-1 overexpression ($P = 0.016$) retained its adverse prognostic role. For the clinicopathologic factors, age ($P = 0.15$), tumor size ($P = 0.50$), and nodal status ($P = 0.10$) were also significant in this multivariate analysis, whereas estrogen receptor ($P = 0.60$), progesterone receptor ($P = 0.99$), HER2 ($P = 0.94$), TNM stage ($P = 0.72$), grade ($P = 0.49$), and menopausal status ($P = 0.19$) were not significant. Because p-p38 was also significant by univariate analysis, we did a second multivariate analysis, including MKP-1, p-p38, and all the clinicopathologic factors mentioned above. In this analysis, MKP-1 again retained its independent value ($P = 0.015$), whereas p-p38 lost the significance ($P = 0.13$). None of the patients with tumors nonoverexpressing MKP-1 had died at the time of analysis, thus precluding comparisons of overall survival.

Discussion

We report that in human breast cancers, exposed *ex vivo* to doxorubicin, MKP-1 is a regulable enzyme, and in the majority of specimens, doxorubicin significantly reduced its expression. This event was coupled with phosphorylation of JNK and

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ERK1/2, thus resembling the pattern reported in breast cancer cell lines. Results in a range of breast lesions also pointed to MKP-1 overexpression as a common event during breast malignant transformation. Finally, MKP-1 overexpression was an independent adverse prognostic factor in breast cancer patients.

Induction of MKP-1 by chemotherapeutic drugs, such as cisplatin or alkylating agents, and proteasome inhibitors limits their cytotoxicity, mainly by inactivation of JNK (3, 4, 14, 15, 44). However, other agents, such as doxorubicin (15) or aplidin (25), repress MKP-1, and this is viewed as part of their antitumor mechanisms of action by allowing a more efficient induction of p-JNK. We confirmed that doxorubicin decreased in a concentration- and time-dependent manner MKP-1 transcripts and protein levels in breast cancer cells. In all the cell lines tested, doxorubicin induced p-JNK and p-ERK1/2. The role of p-JNK as a mediator of apoptosis is well established, whereas the consequences of induction of p-ERK1/2 by doxorubicin are less clear. Although p-ERK1/2 is generally coupled with cell proliferation, a persistent p-ERK1/2 induction may be linked to apoptosis (45). It has to be noted that MKP-1 down-modulation is not the sole mechanism of ERK1/2 activation by doxorubicin. In this regard, the generation of oxygen free radicals by doxorubicin is involved in ERK1/2 activation (29). p-p38 increased in four of the six lines tested. It is possible that in some breast cancer cells, p38 activity is minimally affected by MKP-1 (18).

We evaluated doxorubicin effects, added *ex vivo*, on MKP-1 in 50 fresh human breast cancers (30, 31). Doxorubicin induced a significant decrease in MKP-1 and an increase in p-JNK and p-ERK1/2. In contrast, p-p38 did not change significantly. This pattern resembles the one observed in breast cancer cell lines. In another study, dexamethasone induced MKP-1 and inhibited p-ERK1/2 and p-JNK. However, p-p38 levels were not significantly affected (18). These observations indicate that the correlation between MKP-1 and p-ERK1/2, p-JNK, and p-p38 in breast cancer is not always straightforward. This variability may be related to doxorubicin effects on MAPKs other than via MKP-1 (16), and also because depending on cell type and context, MKP-1 might dephosphorylate and inactivate any of the three MAPKs (4, 5). Interestingly, only in the tumors that down-modulated MKP-1 level there was a significant increase in p-ERK1/2 and p-JNK. Furthermore, doxorubicin decreased proliferation rates only in tumors with MKP-1 down-modulation (data not shown). Full data on proliferation will be reported in a separate methods manuscript.[9] There was a subset of tumors with high basal MKP-1 expression in which doxorubicin did not down-regulate, or slightly increased, MKP-1, for as yet unknown reasons. Of note, in breast cancer cells genetically expressing high, irrepressible levels of MKP-1, the ability of doxorubicin to enhance the cytotoxicity of alkylating agents was lost (15). Regardless of mechanisms, we hypothesized that lowering the level of MKP-1 might enhance the ability of doxorubicin to increase its cytotoxicity. Supporting this notion, BT-474 cells pretreated with Ro-31-8220, an agent that reduces MKP-1 expression, were more sensitive to doxorubicin. This finding agrees with an enhanced cytotoxicity of doxorubicin in breast cancer cells with genetic suppression of MKP-1 and in MKP-1 knockout mouse embryo fibroblasts (15). However, due to the nonspecific effects of Ro-31-8220, a variety of mechanisms might explain our result (32).

A role of MKP-1 in chemoresistance has been reported for cisplatin in lung and ovarian cancer (43, 44, 46). We have observed an enhancement of cisplatin cytotoxic effects in human non–small cell lung cancer cells when the activity of both MKP-1 and nuclear factor-κB was blocked.[10] MKP-1 is also induced in response to radiation and limits its apoptotic effects (47). Adding to the complexity of the field, other members of the MKP family are also involved in drug resistance. In this regard, MKP-3 is overexpressed in breast cancer and mediates resistance to tamoxifen (48).

To date, there were two reports on MKP-1 expression in human breast cancer (26, 27). In one study, MKP-1 was overexpressed in the early phases of prostate, colon, and bladder carcinogenesis, with progressive loss of expression with higher histologic grade and in metastases (26). In contrast, breast carcinomas showed significant MKP-1 expression even when poorly differentiated or in late stages of the disease. MKP-1 and

Table 1. Patient clinicopathologic characteristics and MKP-1 overexpression

Characteristic (no. patients)	Overexpressing MKP-1, *n* (%)	*P*
Total series of patients (*n* = 96)	43 (45)	
Menopausal status		0.98
Premenopausal (*n* = 13)	6 (46)	
Postmenopausal (*n* = 72)	33 (46)	
Unknown (*n* = 11)	4 (36)	
Infiltrating tumor type		0.12
Ductal (*n* = 78)	32 (41)	
Lobulillar (*n* = 18)	11 (61)	
Tumor grade		0.55
1 (*n* = 24)	10 (42)	
2 (*n* = 46)	19 (41)	
3 (*n* = 26)	14 (54)	
Estrogen receptor		0.59
Negative (*n* = 20)	10 (50)	
Positive (*n* = 76)	33 (43)	
Progesterone receptor		0.08
Negative (*n* = 28)	16 (57)	
Positive (*n* = 68)	27 (40)	
HER2		0.72
Negative (*n* = 77)	33 (43)	
Positive (*n* = 19)	10 (53)	
Primary tumor size		0.45
T1 (*n* = 48)	19 (40)	
T2 (*n* = 37)	17 (46)	
T3 (*n* = 7)	5 (71)	
T4 (*n* = 4)	2 (50)	
Node stage		0.14
N0 (*n* = 57)	22 (39)	
N1 (*n* = 18)	10 (56)	
N2 (*n* = 10)	4 (40)	
N3 (*n* = 6)	5 (83)	
NX (*n* = 5)	2 (40)	
TNM stage		0.22
I (*n* = 42)	15 (36)	
II (*n* = 28)	14 (50)	
III (*n* = 20)	12 (60)	
Unknown (*n* = 6)	2 (33)	

[9] F. Rojo et al., in preparation.
[10] Cortes et al., submitted for publication.

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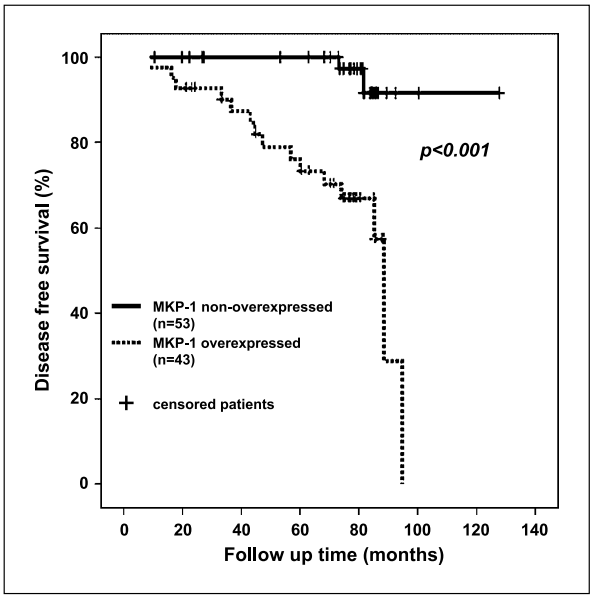

Fig. 5. Actuarial disease-free survival for patients with tumors overexpressing MKP-1 ($n = 43$) versus those nonoverexpressing MKP-1 ($n = 53$), calculated by Kaplan-Meier analysis. See Fig. 3*B* for numerical data of MKP-1 expression in the entire series.

ERK1/2 were coexpressed in most tumors (26). ERK1 enzymatic activity was elevated despite MKP-1 overexpression. No loss of 5q35-ter (containing the MKP-1 locus) was detected by PCR in metastases compared with primary tumors and no mutations were found in the catalytic domain of MKP-1. MKP-1 was overexpressed in advanced disease stages of breast cancer (26, 27). Our work supports the notion that overexpression of MKP-1 occurs during the malignant transformation of the breast (26). Histologically normal breast ductal and lobular epithelial cells typically exhibited faint and diffuse MKP-1 nuclear staining. In contrast, in *in situ* carcinoma, MKP-1 was almost uniformly overexpressed. In infiltrating carcinoma, MKP-1 overexpression occurred in ~50% of the specimens. Notably, in some specimens, there was overexpression of MKP-1 in the *in situ* carcinoma, whereas it was not overexpressed in the infiltrating areas. This finding agrees with a prior study (26) and is also similar to the pattern reported for HER2 (49). In infiltrating breast cancers, several adverse clinicopathologic features were more frequent in MKP-1–overexpressing tumors, but there were no statistically significant associations. We also assessed the interplay between MKP-1 and phospho-MAPKs. A significant association was found between MKP-1 overexpression and low p-p38 as well as with high p-ERK1/2. Similarly to our findings, high expression and activity of ERK has been reported in MKP-1–positive breast cancers, suggesting that ERK might be driving, at least in part, MKP-1 expression in unperturbed conditions (10, 26). Decreased activity of JNK1 has been also observed in breast cancers with MKP activity (27). We did not find statistically significant differences in p-JNK, albeit median Hscores were numerically lower in tumors overexpressing MKP-1.

Studies on the prognostic role of MKP-1 in human malignancies are as yet limited. In ovarian cancer, MKP-1 overexpression was associated to a shorter time to disease progression (50). In contrast, in non–small cell lung cancer, MKP-1 overexpression was linked to an improved prognosis (51). The possible reasons to explain this difference include diverse roles of MKP-1 in different malignancies, methodologic issues, or differences inherent to each population study. Here, we report an association between MKP-1 overexpression and high risk of breast cancer relapse by univariate and multivariate analysis. Taking all these considerations into account, it seems that the prognostic role of MKP-1 should be further assessed in larger series with clearly defined patient populations.

In summary, overexpression of MKP-1 occurs during the malignant transformation of the breast and was an independent adverse prognostic factor. Furthermore, MKP-1 is a regulable enzyme in human breast cancers. MKP-1 repression by doxorubicin was associated with a significant increase of phospho-MAPKs, including ERK1/2, JNK, and, in a nonsignificant manner, p-p38. This finding, in clinical specimens, is consistent with the biological role of MKP-1 and may underlay, at least in part, the cytotoxic effects of doxorubicin. Taken together, we believe that these results justify further studies of MKP-1 as a potential target or marker in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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EDUCATIONAL SERIES Green Series*

The role of the NFκB signalling pathway in cancer

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Abstract The nuclear factor kappa B (NFκB) signalling pathway regulates the expression of hundreds of genes that are involved in different cellular processes such as cell proliferation, survival, stress responses, cellular immunity and inflammation. Its aberrant regulation is involved in several pathologies, but its relevance in cellular transformation and cancer development has been extensively studied. Mutations in the core components of NFκB as well as in the cellular machinery that regulates its activation have been found in many types of tumours. On the other hand, its role in promoting cell survival is an important obstacle in many cancer therapies. The development of chemical inhibitors that block NFκB activation acting either directly on IKKs or on the proteosome machinery has shown antitumour and proapoptotic activity both in preclinical and clinical studies.

Keywords rel · NFκB · IκB · Bortezomib · IKK

The nuclear factor κB (NFκB) signal transduction pathway is a complex network that regulates a cellular pathway that controls the expression of hundreds of genes involved in a myriad of physiological and pathological scenarios. Active NFκB transcription factors are dimeric and composed of a combination of members of the rel transcription factor family. Five different rel family members have been identified in mammals: relA (p65), relB, c-rel, NFκB1 (p50 and its precursor p105) and NFκB2 (p52 and its precursor p100) (Fig. 1). This family can associate in homo- or heterodimer combinations by virtue of their rel Homology domain (RHD), which contains sequences required for DNA binding, dimerisation, nuclear localisation and inhibitor (IκB) binding. These combinations are retained inactive in the cytoplasm, by binding with the inhibitory family of proteins IκBs [1]. There are two subfamilies in the NFκB transcription factors: the NFκB and the rel family. The NFκBs p105 and p100 are distinguished by the C-terminal domains, which include an IκB-like inhibitory domain that contains several copies of the ankyrin repeat (ANK). As a consequence, these proteins are synthesised as inactive precursor proteins (p100 and p105) that are processed by a proteasome-mediated proteolysis to their mature forms, able to bind DNA, p52 and p50 respectively. The rel subfamily of proteins (c-rel, relA and relB) are not proteolytically processed and contain C-terminal transactivation domains that are unrelated to one another.

NFκB and rel homo- or heterodimers can bind to their target sites on DNA, called κB sites. However the affinity of individual subunits to one another or to DNA binding sites can be variable. The regulation of NFκB degradation involves a family of IκB kinases (IκKs) and the ubiquitin E3 ligase SCF/bTrCP.

Canonical and non-canonical NFκB signalling pathways

Two different signalling pathways are involved in NFκB activation [2] (Fig. 2). The activation events that modulate these pathways are mediated by a family of kinases called

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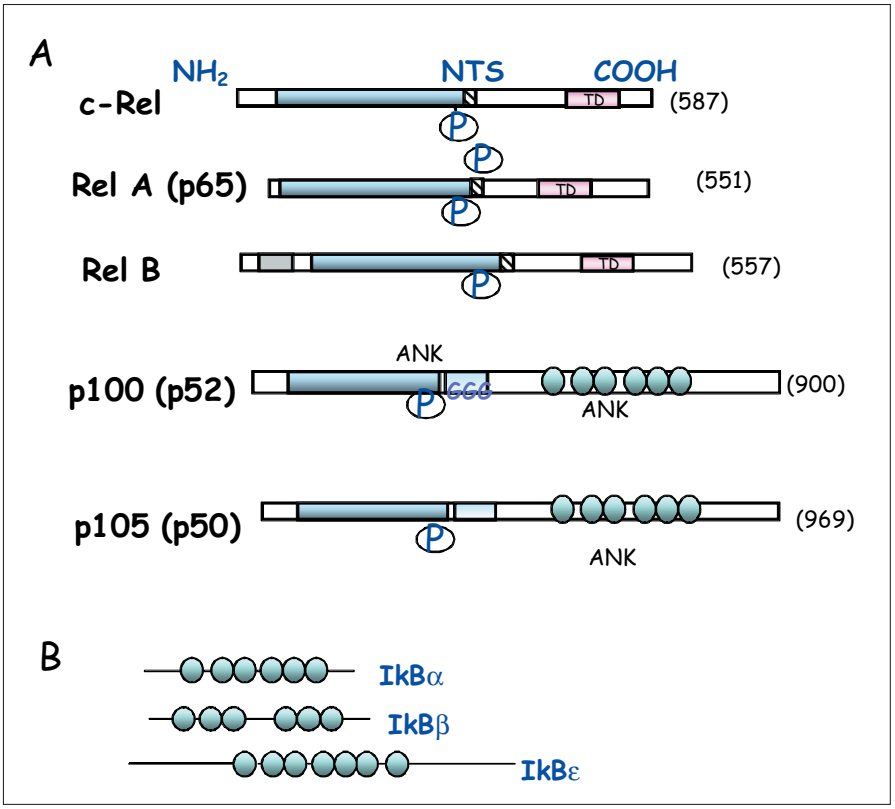

Fig. 1 The mammalian members of the NFκB family. **a** The NFκB family. In mammalian cells there are three NFκB members. The rel family: c-rel, p65/relA and relB. The NFKB2 family p105 and p100 give rise to the mature forms p50 and p52 respectively. **b** The IκB family. The inhibitor κB family consists of IκBα, IκBβ and IκBε. Like p105 and p100, the IκB proteins contain ANK motifs in their C termini

the IκKs (inhibitory kappa kinases). The IκK complex consists of three core subunits, the catalytic subunits IκKα and IκKβ (also known as IκK1 and IκK2) and several copies of a regulatory subunit called NFκB essential modifier (NEMO, also known as IκKγ). The canonical pathway is induced mainly by IκB kinase β (IκKβ) thorough phosphorylation of either the three canonical IκB proteins, mainly IκBα but also IκBβ and IκBε. This phosphorylation signals degradation of the protein by the 26S proteosome.

The non-canonical pathway is activated thorough IκKα and by the NFκB inducing kinase (NIK) and utilises the p52 precursor protein p100. p100 is processed by the 26S proteosome. p52/relB heterodimers are activated by the non-canonical pathway and have higher affinity for distinct κB elements and might regulate expression of a more specific set of NFκB genes than those activated by the canonical pathway.

Activators of the canonical pathway include various inflammatory stimuli, including pro-inflammatory cytokines, tumour necrosis factor α (TNFα) and interleukin 1 (IL-1), engagement of the T-cell receptor (TCR) and exposure to bacterial products such as lipopolysaccharides (LPS) [3]. Other activation mechanisms of the canonical pathway are also genotoxic stimuli such as ionising radiation or some therapeutic drugs that induce DNA damage.

The non-canonical pathway is activated by stimuli such as the CD40 and lymphotoxin-β-receptors, B-cell-activating factor of the TNF family (BAFF), LPS and latent-membrane protein (LMP-1) or Epstein-Barr virus [2, 4].

The canonical pathway has been involved in development, immunity and cancer development. On the other hand, the non-canonical pathway regulates survival of premature B-lymphocytes and development of peripheral lymphoid tissues. Different pathogens, growth factors, cytokines and carcinogens that induce activation of the canonical pathway (p50/relA) have been involved in cancer progression.

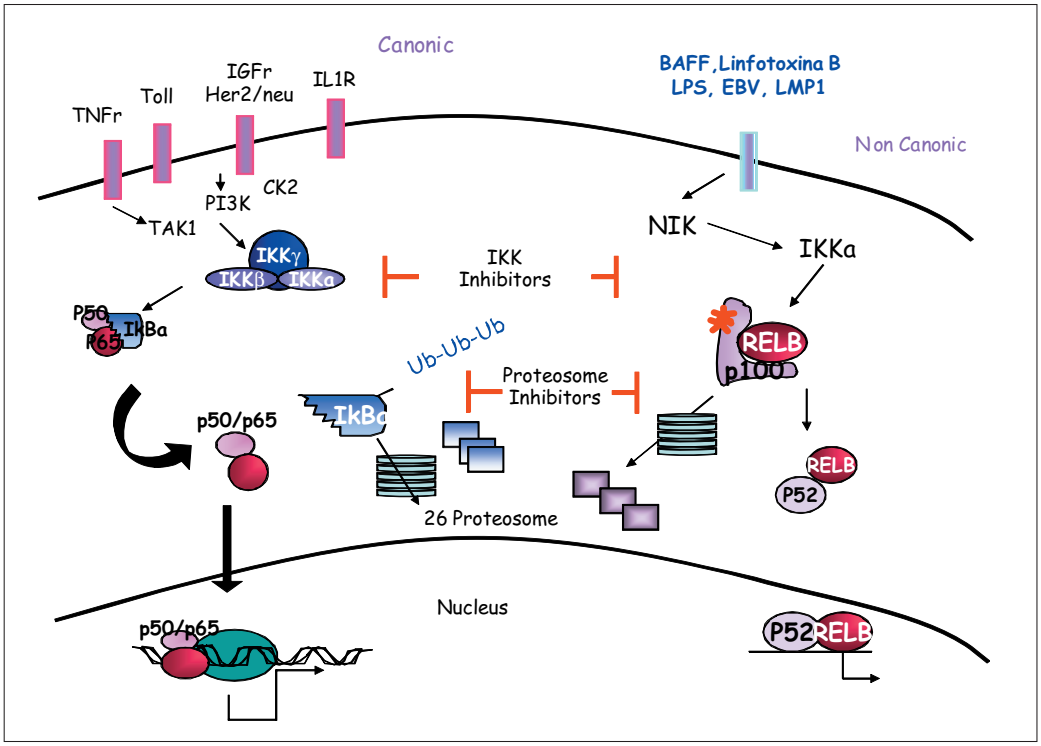

Fig. 2 NFκB signalling pathways. The canonical pathway is modulated mainly by IκB through phosphorylation of IκBα that signals degradation of the protein by the 26S proteosome. The IκK complex consists of three core subunits, the catalytic subunits IκKα and IκKβ and the regulatory subunit IκKγ. The non-canonical pathway is activated through IκKα and by NIK, which phosphorylates p100 and is processed by the 26S proteosome. Red highlights inhibitors of the kinases and the proteasome

Genetic alterations of the NFκB signalling pathway in cancer

Different modifications in the sequence, due to mutations, gene deletions or genetic rearrangements have been found in different members of this signalling pathway in different tumours. We will summarise here the more relevant ones.

Rel gene alterations

The human REL gene is the most frequently altered among the NFκB signalling pathway. The genetic alterations are amplifications, deletions, point mutations and occur more frequently in B cells [5]. Rel gene amplifications are related mainly to Hodgkin's lymphoma (HL) and non-Hodgkin's B-cell lymphomas [6]. The amplification includes REL and several other genes, but REL is the only gene that overlaps in these lymphoma-associated amplifications [6]. The mechanism by which c-rel induces oncogenesis is a consequence of the increasing gene copy number that saturates and overcomes the IκB inhibitory system. Different experimental evidence suggests that overexpression of rel leads to enhanced mature B-cell proliferation and survival. Indeed, rel target genes have been found to be overexpressed in mono-diffuse large-B-cell lymphomas. Furthermore, rel mRNA expression has been associated with poor prognosis in splenic marginal B-cell lymphomas. Accordingly, inhibition of rel expression by SiRNA or chemical inhibitors has been shown to block large B-cell lymphoma growth.

REL gene rearrangements or deletions have been detected very rarely in human lymphomas and include REL gene translocated to a position near the light chain enhancer, a truncated REL gene near the C-terminus and integration of Epstein-Barr virus near to REL, which resulted in increased REL expression [7]. Point mutations, (ser/pro) located into the transactivation domain of rel (ser525) have been recently detected in two human B-cell lymphomas. In all cases increased levels of REL-activated genes are found in the tumours.

NFκB2 gene alterations

Alterations in the NFκB2 locus have been found in 2% of human B- and T-cell leukaemias and lymphomas [8]. These changes included structural alterations in sequences of the ANK repeats that are removed, but the p52 sequence remains unaltered. These truncated p100 proteins (p52) are often overexpressed, whereas the p100 encoding allele is often deleted or not expressed. These truncated p100 proteins have been shown to be weakly oncogenic in mouse fibroblast.

Alterations in genes encoding IκB

Inactivating mutations in the IκB gene have been found in HL. Mutations have been found in Hodgkin Reed-Sternberg (HRS) cells and retroviral insertions have been found in mouse lymphomas. These mutations increase IκB turnover and consequently no IκB proteins are detected. As a consequence there is a constitutive NFκB signalling and several NFκB target genes are overexpressed, including those encoding anti-apoptotic and growth factor promoting proteins [9].

Increased expression of BCL3

BCL3 encodes an IκB-like protein that serves as a co-activator for p50 and p52 homodimers. Some patients with B-cell CLL have a chromosomal translocation in which BCL3 gene becomes positioned 3´ to the switch region or the immunoglobulin heavy chain gene. Such overexpression of BCL3 is expected to result in increased transcription of genes normally regulated by p52 or p50 homodimers [10].

Constitutive activation of NFκB

Numerous studies of the literature have described, either in cell lines or in tumour samples, a constitutive activation of the NFκB pathway, and this has been related either to tumorigenesis or therapy resistance. This is the case for human lymphomas, as described above, and also for carcinomas of head and neck, oesophagus, pancreas, prostate, lung, colon and cervix. Besides modifications on the members of the NFκB sequence itself, modifications of the upstream molecules that regulate activation plays an important role in tumorigenesis. Overexpression of different tyrosine kinase receptors, such as epidermal growth factor (EGF), Her2/neu, hepatocyte growth factor (HGF) and others such as IL1 results in activation of the NFκB pathway [11]. The participation of these growth factor receptors varies among different tumours. Involvement of EGFr and Her2/neu signalling has been demonstrated in activation of NFkB in breast cancer where is mediated by activation of PI3-kinase and IKK. In head and neck cancer, activation of EGFr, IL1, PI3Kinase, akt and IKK mediate activation of NFκB. For prostate carcinomas, activation of HGF, PI3K and IKK mediate NFκB signalling. Likewise, activation of IKK and CK2 is observed in colon carcinoma.

On the other hand, many genes involved in cell proliferation, tumorigenesis, cell migration and survival are activated by NFκB. Targets of NFκB are genes such as cyclin D1, BclXl, inhibitors of caspases (IAPs), GRO1, IL8, Bax, Bcl2 and VEGF. In short, genes whose expression is activated by NFκB contribute to the transformed phenotype in human tumours by controlling both proliferation and survival [12–14]. Furthermore, as some chemotherapeutic agents also induce an activation of NFκB, the outcome of different therapies would be influenced by both the basal NFκB activation on each tumour and that resultant from the elected therapy.

Inhibition of NFκB in cancer treatment

Different strategies have been implemented in order to inhibit basal NFκB activation, because of the relevance of this process in cancer and other human pathologies, such as those related with inflammation.

Proteosome inhibitors

Proteosome regulates the degradation of IκB and hence inhibits NFκB, as well as degradation of other cellular proteins. The proteasome inhibitor bortezomib has been shown to have clinical activity and has been approved for the treatment of multiple myeloma [15]. Multiple myeloma cells are very sensitive to NFκB inhibition and bortezomib has NFκB-dependent and -independent activity on these cells. A limited activity of this drug as a single therapy has been described in solid tumours such as metastatic melanoma, colon, breast, prostate and renal cell carcinomas, as well as for refractory cutaneous T-cell lymphoma [16–21].

Evidence has also been obtained of clinical activity with different combinations of bortezomib and other agents. Phase I trials have been conducted with bortezomib in combination with carboplatin in patients with platinum- and taxane-resistant ovarian cancer [22], and also for non-small-cell lung cancer in combination either with pemetrexed [23] or gemcitabine and carboplatin [24]. Results of these studies have been used to design phase II trials. The efficiency of bortezomib in combination with targeted therapies in solid malignancies is being studied in phase II trials.

IKK inhibitors

Preclinical evidence, using knock-out models, indicates that inhibition of IKKβ and p65 sensitises cells to apoptosis in response to different therapies. Two antagonists of

IKKβ that efficiently inhibit IκB degradation have been extensively studied at the preclinical level. BMS-345541 (Bristol Myers, Princeton, NJ) has show apoptotic and antitumour activity in melanoma xenograft models [25]. PS-1145 or ML120b (Millennium Pharmaceuticals) has shown activity in multiple myeloma cells, chronic myelogenous leukaemia, diffuse large B-cell lymphoma and prostate carcinoma cells [26–28]. The range of activity of these compounds is at a micromolar level but no clinical studies have been carried out with these inhibitors.

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ANEXO II

Currículum Vitae

CURRICULUM VITAE

María Cortés Sempere

INFORMACIÓN PERSONAL

Dirección: C/ Martin Luther King 54, 9 1ºA, Alcorcón (Madrid)
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Fecha de nacimiento y lugar de nacimiento: 28 de Febrero de 1981 Madrid
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FORMACIÓN ACADÉMICA

- 2005 / actualidad: Realizando la tesis doctoral en el Instituto de Investigaciones Biomédicas de Madrid
- Octubre del 2008: Diploma de estudios avanzados (DEA). Universidad Autónoma de Madrid
- Septiembre del 2004: Licenciada en bioquímica por la Universidad Autónoma de Madrid

PARTICIPACIÓN EN PROYECTOS DE INVESTIGACIÓN

2005-2007
Como miembro del equipo investigador:

Título del proyecto: Identification of genes involved in cisplatinum chemotherapy acquired resistance in NSCLC.
Entidad Financiadora: Fundación MUTUA Madrileña
Duración desde: Abril 2007 hasta: Abril 2010
Investigador Principal: Dra. Inmaculada Ibáñez de Cáceres

Título del proyecto: Papel de NFκB e inhibidores del protesoma en el tratamiento de cáncer no microcítico de pulmón
Entidad Financiadora: Fondo de investigación sanitaria (FIS 05/1305)
Duración desde: 2005 hasta: 2008
Investigador Principal: Dra. Rosario Perona Abellón

2003-2004
Como estudiante universitaria en prácticas:

Desarrollo de herramientas moleculares para el empleo de E2F1 en terapia antitumoral
Instituto de Investigaciones biomédicas “Alberto Sols” CSIC-UAM
Responsable del Proyecto: Dr. Miguel Campanero

EXPERIENCIA y TÉCNICAS

Experiencia técnica adquirida en Cultivos Celulares.

- Cultivo de líneas celulares (Líneas celulares tumorales)

Experiencia técnica adquirida en Técnicas de Biología Molecular.

- Transformación bacteriana y purificación de plásmidos: Digestión por enzimas de restricción
- Técnica de transfección celular por fosfato cálcico y lipofectamina de manera transitoria o estable.
- Estudio de expresión de genes por RT-PCR y Q-PCR.
- Análisis de la expresión de genes en líneas celulares por ensayos de reportero.
- Aislamiento de ARN. Técnicas de clonaje por RT-PCR.
- Medida de la proliferación celular por tinción de cristal violeta.
- Técnica de clonaje de genes a partir de RNA celular y librería genómica.
- Citometria de Flujo
- Ensayos de movilidad en gel.
- Inmunodetección de proteínas
- Ensayos de inmunofluorescencia

Experiencia técnica adquirida con Ensayos Animales

- Inducción subcutánea de tumores y tratamiento de los mismos

CONGRESOS

- **XIII National congress, Spanish association for cancer research(ASEICA), Salamanca, Spain. Septiembre 2011**

- ***21th Meeting,* EACR. Oslo, Norway. 2010**
  Presentación del poster: IGFBP-3 promoter methylation is responsible of chemotherapy resistance to CDDP through the activation of the PI3K/Akt intracellular signaling pathway. Maria Cortes Sempere, de Miguel, Pernía O, Javier de Castro Carpeño, Manuel Nistal, López-Ríos, Cristobal Belda-Iniesta, Rosario Perona and Inmaculada Ibañez de Caceres.

- Simposio Internacional “Proteínas de la superfamilia Ras: regulación, moléculas efectoras y participación en enfermedades humanas” Centro de Investigación del Cáncer de Salamanca, 18 y 19 de Mayo de 2009.

- **XII National congress, Spanish association for cancer research(ASEICA), Madrid, Spain. May 2009**
  Presentación del poster: *Signalling pathways controlling MKP1 expression in cancer.* **M. Cortés Sempere,** I. Ibanez de Cáceres, C. Belda-Iniesta, L. Sastre and R. Perona.

  Presentación del poster: Identification of DNA methilation markers as novel predictors and targets for the therapeutic response to cisplatin in NSCLC.

Ibanez de Cáceres, **M. Cortés,** C. Moratilla, R. Machado-Pinilla, V. Rodríguez Fanjul, C. Manguán, J. Castro, M. Nistal, C. Belda-Iniesta and R. Perona.

- ***20th Meeting,* EACR. Lyon, France. 2008**
  Presentación del poster :Differential expression of DNAmethyltransferase in sensitive versus cisplatin resistant NSCLC cell lines. EACREJC2008, 6(9):107.

  Cortés M, Perona R, and Ibáñez de Cáceres I.

  Presentación del poster: Identification of drug-sensitive prediction genes by an epigenetic reactivation screen o CDDP-resistant NSCLC cell lines EJC2008, 6(9):106-107. Ibañez de Caceres I, **Cortes M,** Moratilla C, MachadoPinilla R, Rodríguez-Fanjul V, Manguan C, De-Castro-Carpeno J, Belda-Iniesta C, NistaL M and R Perona.

- **XI National congress, Spanish association for cancer research (ASEICA). Las Palmas, Spain, May 2007**
  Presentación del poster : Establishment and expression profile of the Cisplatin resistant H23 and H460 NSCLC cell lines. Ibáñez de Cáceres I, **María Cortés-Sempere,** Carmen Moratilla and Rosario Perona.

- **X National Congress, Spanish association for cancer research (ASEICA). Pamplona, Spain. Oct 2005.**
  Critical pathway controlling cellular response towards cisplatin in non-small cell lung cancer (NSCLC). **M. Cortes,** J.Albanell, S.Chattopadhyay, M. Tapia, A. Rovira, C. Belda Iniesta, J. De Castro, C. Maguán García, R. Machado- Pinilla, M. Gonzalez Barón and R. Perona.

CURSOS

- **Curso capacitación de Operadores de instalaciones radioactivas (laboratorio con Fuentes no encapsuladas).** Servicio de protección radiológica. Instituto de investigaciones biomédicas de Madrid. Febrero de 2007.

- **Curso básico de radioactividad.** Instituto de investigaciones biomédicas de Madrid. Abril de 2005.

PUBLICACIONES

- **IGFBP-3 methylation-derived deficiency mediates the resistance to cisplatin through the activation of the IGFIR/Akt pathway in non-small cell lung cancer. Cortés-Sempere M,** de Miguel MP, Pernía O, Rodriguez C, de Castro Carpeño J, Nistal M, Conde E, López-Ríos F, Belda-Iniesta C, Perona R, de Caceres II.
  Oncogene. 2012 Apr 30. doi: 10.1038/onc.2012.146. [Epub ahead of print]

- **MicroRNAs as novel epigenetic biomarkers for human cancer.**
  Cortés-Sempere M, Ibáñez de Cáceres I.
  Clin Transl Oncol. 2011 Jun;13(6):357-62.

- **IGFBP-3 hypermethylation-derived deficiency mediates cisplatin resistance in non-small-cell lung cancer.**
  Ibanez de Caceres I, **Cortes-Sempere M,** Moratilla C, Machado-Pinilla R, Rodriguez-Fanjul V, Manguán-García C, Cejas P, López-Ríos F, Paz-Ares L, de Castrocarpeño J, Nistal M, Belda-Iniesta C, Perona R.
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- **MKP1 repression is required for the chemosensitizing effects of NF-kappaB and PI3K inhibitors to cisplatin in non-small cell lung cancer.**
  Cortes-Sempere M, Chattopadhyay S, Rovira A, Rodriguez-Fanjul V, Belda-Iniesta C, Tapia M, Cejas P, Machado-Pinilla R, Manguan-García C, Sánchez-Pérez I, Nistal M, Moratilla C, de Castro-Carpeño J, Gonzalez-Barón M, Albanell J, Perona R.
  Cancer Lett. 2009 Dec 28;286(2):206-16. Epub 2009 Jun 23.

- **Mitogen-activated protein kinase phosphatase-1 in human breast cancer independently predicts prognosis and is repressed by doxorubicin.**
  Rojo F, González-Navarrete I, Bragado R, Dalmases A, Menéndez S, Cortes-Sempere M, Suárez C, Oliva C, Servitja S, Rodriguez-Fanjul V, Sánchez-Pérez I, Campas C, Corominas JM, Tusquets I, Bellosillo B, Serrano S, Perona R, Rovira A, Albanell J. Clin Cancer Res. 2009 May 15;15(10):3530-9. Epub 2009 May 5.

- **The role of the NFkappaB signalling pathway in cancer.**
  Cortés Sempere M, Rodríguez Fanjul V, Sánchez Pérez I, Perona R.
  Clin Transl Oncol. 2008 Mar;10(3):143-7. Review.

IDIOMAS

Inglés: nivel medio hablado y escrito

INFORMÁTICA

Conocimiento a nivel usuario:

- Windows
- Procesadores de Texto: Microsoft Word
- Hojas de Cálculo: Excel
- Gráficos: Microsoft Powerpoint, AdobePhotoshop